Charles University in Prague
Faculty of Science

Terrestrial algae of the genus *Klebsormidium* (Streptophyta) in the light of the hypothesis „Everything is everywhere, but the environment selects“

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Ph.D. Thesis

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Prague, 2016

Declaration

I hereby declare that I have written this thesis independently, using the listed references; or in cooperation with other paper co-authors. I have submitted neither this thesis, nor any of its parts, to acquire any other academic degree.

Prague, 31st May 2016

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David Ryšánek

Acknowledgements

I would like to thank Pavel Škaloud, who has been my supervisor since my bachelor study. During this time, he had a lot of work with me and every time I needed, he gladly helped me. Thanks to him I enjoyed this work very much.

I would like to thank all co-authors, mainly Andreas Holzinger and Fabio Rindi, for their big help during the research and preparing manuscripts.

Thank you my colleagues from the phycology research group in Prague for friendly and inspiring atmosphere. Special thanks go to all of you who read the previous versions of the thesis or its summary. Your comments and ideas were really helpful.

Finally, I would like to thank my parents, because without their support it would be very difficult to study Ph.D. and finish it.

And also I would like to thank those who are close to me for their friendship and support.

The Charles University Science Foundation provided funding of this work, project GAUK 1544214. The research stays was supported by the Czech-Austrian project AKTION 65p5. Moreover the study was supported by the Austrian Science Fund (FWF) Project P 24242-B16 and FWF project I 1951-B16 to AH. Finally, I would like to thank Nadace "Nadání Josefa, Marie a Zdeňky Hlávkových" for their money support at the conference in London and Moscow.

This thesis is based on the following five papers:

1. Škaloud P., Lukešová A., Malavasi V., **Ryšánek D.**, Hrčková K. and Rindi F. (2014) Molecular evidence for the polyphyletic origin of low pH adaptation in the genus *Klebsormidium* (Klebsormidiophyceae, Streptophyta). *Plant Ecology and Evolution* 147: 333-345.

2. **Ryšánek D.**, Hrčková K. and Škaloud P. (2015) Global ubiquity and local endemism of free-living terrestrial protists: phylogeographic assessment of the streptophyte alga *Klebsormidium*. *Environmental Microbiology* 17: 689-698.

3. **Ryšánek D.**, Elster J., Kováčik L. and Škaloud P. (2016) Diversity and dispersal capacities of a terrestrial algal genus *Klebsormidium* (Streptophyta) in polar regions. *FEMS Microbiology Ecology* 92.4: fnw039.

4. **Ryšánek D.**, Holzinger A. and Škaloud P. (2016) Influence of substrate and pH on the diversity of aeroterrestrial alga *Klebsormidium* (Klebsormidiales, Streptohyta): A potentially important factor for sympatric speciation. *Phycologia* 55: 347-358.

5. Rindi F., **Ryšánek D.** and Škaloud P. (2016) Problems of epitypification in morphologically simple green microalgae: a case study of two widespread species of *Klebsormidium* (Klebsormidiophyceae, Streptophyta). *Fottea* (submitted)

Authors’ contributions:

Paper 1. Fabio Rindi with Pavel Škaloud designed the study. Fabio Rindi, Alena Lukešová Kristýna Hrčková and Veronica Malavasi collected and cultivated all strains. David Ryšánek made all molecular works. Pavel Škaloud and Fabio Rindi jointly wrote the manuscript.

Paper 2. Pavel Škaloud and David Ryšánek jointly planned the study. Pavel Škaloud collected the samples in the Czech Republic, Japan and Washington and sequenced the strains from the Czech Republic. David Ryšánek collected samples in Wales, isolated strains from samples from Wales, Japan, and Washington and sequenced the strains. He also performed

phylogenetic analyses together with Pavel Škaloud. Kristýna Hrčková collected and sequenced strains from Ohio and Connecticut. David Ryšánek wrote the manuscript and Pavel Škaloud contributed to improving the text of the manuscript.

Paper 3. David Ryšánek, Pavel Škaloud, and Josef Elster jointly planned the study. Josef Elster and Lubomir Kovačik collected and isolated almost all strains from polar regions. David Ryšánek collected and isolated some additional strains from Svalbard (during the 2014 expedition) and from temperate zone. He also made all molecular works and together with Pavel Škaloud executed phylogenetic analysis. David Ryšánek wrote the manuscript and Pavel Škaloud contributed to improving the text of the manuscript.

Paper 4. David Ryšánek, Pavel Škaloud, and Andreas Holzinger jointly planned the study. David Ryšánek collected the strains, made molecular works and all ecophysiological measurements. Pavel Škaloud made the statistical analysis. David Ryšánek wrote the manuscript and Pavel Škaloud with Andreas Holzinger contributed to improving the text of the manuscript.

Paper 5. Fabio Rindi with Pavel Škaloud designed the study. Fabio Rindi collected the samples in Padova, Italy. Pavel Škaloud collescted samples in Strasburg, France. David Ryšánek cultivated samples, isolated strains, made all molecular works. Pavel Škaloud and Fabio Rindi jointly wrote the manuscript.

On behalf of all the co-authors, I declare the keynote participation (as first author) of David Ryšánek in completing the research and writing the papers, as described above.

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Pavel Škaloud

Abstract

In 1934, Baas Becking mentioned hypothesis “Everything is everywhere, but the environment selects“, which still contains three current controversial issues in protists: (1) disunity in distribution patterns, (2) species concepts, and (3) the ecological preferences. In recent years, protistan biogeography and diversity has become a highly debated topic. Two opposite hypotheses have been proposed: the ubiquity model, which emphasizes the cosmopolitan distribution of protists with low global diversity; and the moderate endemicity model, which admits the existence of endemic species with limited distribution, leading to relatively high diversity. However, habitat preferences and ecological aspect to the distribution of species are rather missing in the discussion about geographic distribution.

We examined terrestrial algal genus *Klebsormidium* (Streptophyta), which is one of the most abundant microautotrophs in various terrestrial and aerophytic habitats, and further (i) tested whether there is any biogeographical pattern of this microorganism, (ii) examined if there is a comparable diversity in the polar and temperate regions, (iii) investigated a potential role of ecological speciation processes on diversification, and (iv) clarified the taxonomic identity of *Klebsormidium* species.

Our analyses revealed the presence of two different distribution patterns which are supposed to characterize both macroorganisms and protists. We demonstrated an unlimited dispersal and intensive gene flow within one of the inferred lineages (superclade B). However, the majority of *Klebsormidium* clades showed rather limited distribution. In addition, we detected a significant decrease of species richness towards the poles, i.e. the macro-ecological pattern typical of macroorganisms. Species within a single protist genus may thus exhibit highly contrasting distribution patterns, based on their dispersal capabilities, which are usually shaped by both intrinsic and extrinsic factors. In addition, we determined a distinct ecophysiological differentiation among distantly and closely related lineages, thereby corroborating our hypothesis that the sympatric speciation of terrestrial algae is driven by ecological divergence. We clearly showed that pH is a critical ecological factor that influences the diversity of autotrophic protists in terrestrial habitats, and adaptation to low pH conditions has been developed multiple times independently in genus *Klebsormdium*. In the end, based on the original descriptions, we conclude that the designation of the epitype of *K. flaccidum* was correct, whereas the epitype of *K. nitens* (which consists of material collected tens of thousands of km far from the type locality) was most probably incorrect.

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1. INTRODUCTION

1.1. Microbial biogeography

Knowledge of biogeography is necessary for the understanding of evolution, because geographical isolation is a crucial mechanism for the speciation of macroorganismal lineages (O´Malley 2008). Darwin developed his theory of natural selection by a thorough appreciation of biogeographical data, and its implications for the evolution of flora and fauna (Richardson 1981, Browne 1983). For microorganisms, biogeography is usually thought to be irrelevant. In 1934, Lourens Baas Becking encapsulated the idea of cosmopolitan microbial distributions in his famous maxim "Everything is everywhere, but, the environment selects" (Baas Becking 1934, De Wit and Bouvier 2006). That is, all microbe species can reach all locations, but the environmental conditions determine which species are ecologically active and therefore present in numbers, which allow them to be recorded by a microbial ecologist. In recent years, the estimate of protist biogeography and diversity has become a highly controversial topic of modern microbial research (Caron 2009, Gast 2015). Two opposite hypotheses have been proposed: (1) the ubiquity model (Finlay et al. 1996, Finlay 2002), which emphasizes the cosmopolitan distribution of protists, and (2) the moderate endemicity model (Foissner 1999, 2006), which admits the existence of endemic species with limited distribution.

The authors, who support the ubiquity theory, proposed that the small organism size, large population sizes, and high dispersal potential of eukaryotic microorganisms would lead to high gene flow across large geographical scales, resulting in ubiquitous species distribution in suitable environments (Finlay 2002, Fenchel and Finlay 2004). The ubiquity model suggests that microbial taxa of typical protist dimensions (approximately 20 μm in diameter) are capable of global dispersal (Finlay 2002, Wilkinson et al. 2012). Biogeographical barriers do not limit this dispersal (Finlay 2002), which is then driven by random events such as wind, ocean circulation, and transport on the bodies of migrating birds or mammals (Fenchel and Finlay 2004). Consequently, individual protist species occur wherever suitable environmental conditions are available. The ubiquity model predicts a very low probability of local extinction in protist populations (Fenchel and Finlay 2003). The consequence of a very low local extinction coupled with an extremely large population size is high local protist diversity and a low global diversity (Fenchel and Finlay 2003, 2004). In contrast, the macroorganims

should have a low local diversity and a high global diversity (Fig. 1). However, the reported low estimates of total protist species richness (Finlay and Fenchel 1999) contradict the results of several molecular phylogenetic studies showing a large cryptic diversity in eukaryotic microorganisms (Von der Heyden et al. 2004, Šlapeta et al. 2006, Simon et al. 2008).

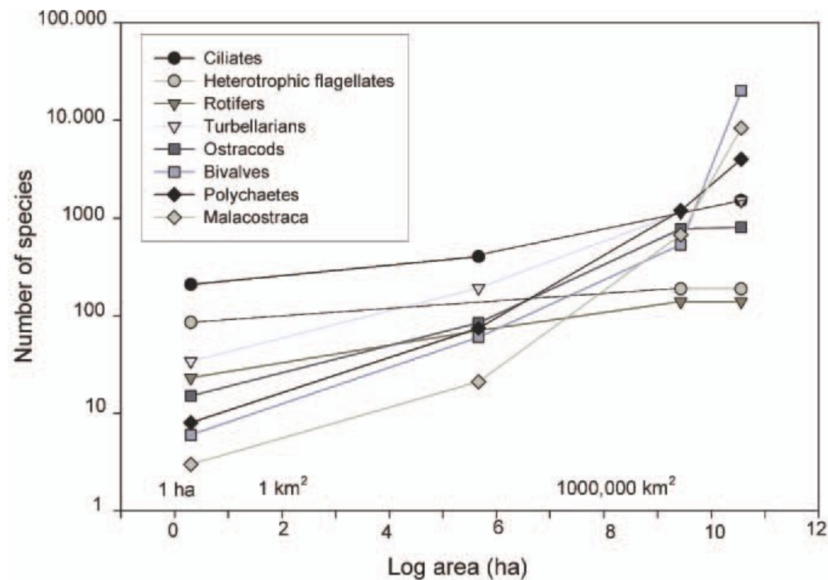

Figure 1. Species–area curves based on the biota from cumulatively larger areas: Nivå Bay, Kiel Bight (Baltic coast, Germany; Gerlach 2000), North-East Atlantic Ocean (including the Barents, Baltic, Mediterranean, and Black Seas; Costello et al. 2001), and the world oceans. From Fenchel and Finlay (2004).

Opponents of the ubiquity theory propose an extraordinarily high global diversity and endemicity of protist species. Foissner (2006) released an information that indicated restricted distributions for several protist groups and promoted the use of flagship species (i.e., species with conspicuous morphologies whose presence/absence could be easily demonstrated in samples) to demonstrate the endemism of several protists. For example, the morphologically distinct desmid species *Micrasterias hardyi* and *Staurastrum victoriense* have been reported only in Australia (Tyler 1996). Accordingly, Foissner and colleagues (Chao et al. 2006, Foissner 2006) proposed a moderate endemicity model, which considers that some protist species have cosmopolitan distributions, whereas other (perhaps rarer) protists have restricted distributions. As protists are very small and thus of low weight, it is widely believed that air currents and animal vectors are the main distribution agents (Schlichting 1960, Maguire 1963, Atkinson 1970, Wilkinson 2001). Wilkinson (2001) showed by a detailed analysis of Arctic and Antarctic testacean communities that species of small sizes (≤ 150 µm) are cosmopolitan.

However, all the data above are in conflict with a simple fact (Foissner 2006, 2008), that mushrooms, mosses, ferns, lichens and horsetails have restricted distributions although their distribution means (spores) are produced in masses and in the size of most protists (≤ 100 µm) (Fig. 2).

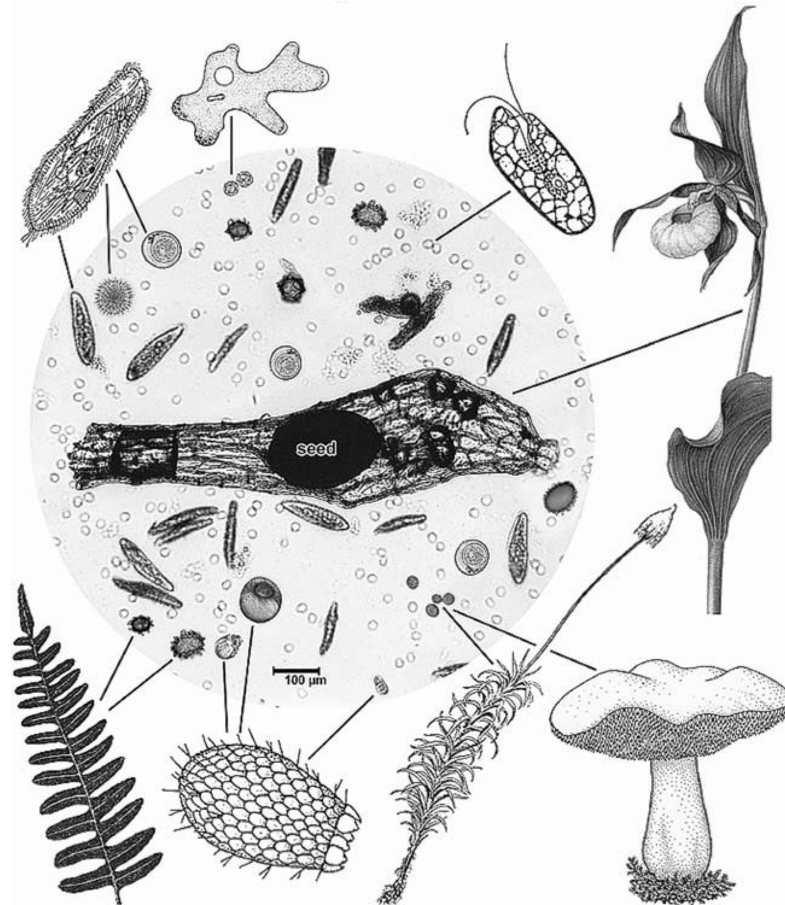

Figure 2. This figure compares, at about the same magnification, trophic and cystic protists (ciliates, Xagellates, naked and testate amoebae) with spores of macrofungi (mushrooms), mosses, ferns, and the minute seed of an orchid (*Vanda caerulescens*). Obviously, all are of minute size and very abundant, for instance, a single *Agaricus campestris* (mushroom) releases 1.6 x 10^{10} spores within 6 days (Webster 1983), which exceeds the abundance of ciliates in a m² of forest soil by several orders of magnitude (Meyer et al. 1989). While nobody denies that mushrooms, mosses, and ferns have biogeographies, protists are widely assumed to be cosmopolitan because their small size and high abundance favour air dispersal, an opinion flawed by this figure. Further, protist cysts lack adaptations for air dispersal, while seeds of many flowering plants have such adaptations, including the orchid seed shown which has wings of large-sized, air-filled cells. From Foissner (2008).

Further, Foissner (2006) also emphasized the importance of biogeographic changes due to human introductions and provided several examples, for instance, the introduction of

the very distinctive alga *Hydrodictyon* to New Zealand along with the imported fish and water plants from East Asia (Kristiansen 1996). Additional impressive cases are, for instance, the introduction of the diatom *Asterionella formosa* to New Zealand (Vanormelingen et al. 2008). Another example is the introduction by ballast water (Nicholls and MacIscaac 2004, Bolch and de Salas 2007). Thus, human-induced biogeographic changes of protist communities are as important as in plants and animals, and should get much more importance in the biogeography debate.

There is a huge evidence for protistan biogeography, comprising multiple lineages such as (1) Stramenopila, e.g., *Spumella* (Boenigk et al. 2005), *Xanthonema* (Rybalka et al. 2009), *Synura* (Škaloud et al. 2014), diatoms (Vyverman et al. 2007, Kooistra et al. 2008, Evans et al. 2009, Veselá et al. 2012); (2) Archaeplastida, e.g., *Micrasterias* (Jurdíková et al. 2014), *Asterochloris* (Řídká et al. 2014, Škaloud et al. 2015), (3) Opisthokonta, e.g., fungi (Taylor et al. 2006), (4) Rhizaria, e.g., *Eocercomonas* and *Paracercomonas* (Bass et al. 2007).

1.2.Diversity

Protist species diversity has been the subject of considerable debate (Finley 2002, Foissner 2006, Caron 2009). Global protist diversity has been proposed to be either extraordinarily high by some (Foissner 1999) or generally much lower and fundamentally different from the biodiversity of macroorganisms by others (Fenchel and Finlay 2003). Accordingly, while Finlay and Fenchel (1999) estimated that there are approximately 20,000 protist species, Foissner (1999) estimated that there might be 30,000 species of ciliates alone; others estimate that there are even several millions of undescribed protist species (Fig. 3) (Pawlowski et al. 2012). In general, the undiscovered species diversity among protists may be orders of magnitude greater than previously thought. The overall species diversity in environmental protist populations is still largely unknown, because most genetic studies are carried out on isolated, clonal cultures. Nevertheless, the growing number of environmental studies of eukaryotic diversity, often based on the Next Generation Sequencing (NGS) data, gives us a better insight into the real diversity of protist species (e.g. Behnke et al. 2011, Edgcomb et al. 2011, Lecroq et al. 2011, Logares et al. 2012). Such substantial differences are primarily caused by differences in methodology (see above), and species concept (De Queiroz 2007, Caron 2009).

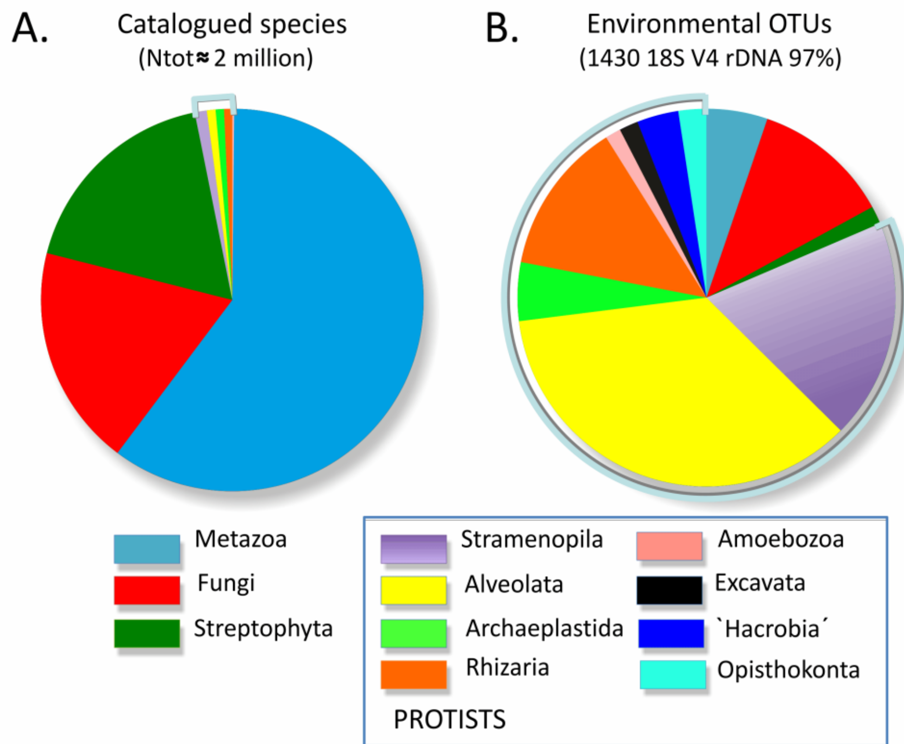

Figure 3. Morphological versus genetic views of total eukaryotic diversity. (A) Relative numbers of described species per eukaryotic supergroup (B) Relative number of V4 18S rDNA Operational Taxonomic Units (97%) per eukaryotic supergroup, based on 59 rDNA clone library surveys of marine, fresh-water, and terrestrial total eukaryotic biodiversity. From Pawlowski et al. (2012).

Another consequence of the ubiquity hypothesis is that, as a result of global dispersal, latitudinal gradients in diversity should be weak or absent once ecological controls are factored out (Hillebrand and Azovsky 2001, Finlay and Fenchel 2004). However, there are only a few studies testing the presence of latitudinal gradients in eukaryotic microorganisms, moreover they are largely incongruent. Whereas Hillebrand and Azovsky (2001) showed that latitudinal gradients of species richness are largely absent for diatoms and presumably also for other unicellular and small multicellular organisms, the studies of Vyverman et al. (2007) and Siver and Lott (2012) showed these gradients on freshwater diatoms and silica-scaled chrysophytes, respectively.

1.3.Species concepts

A definitive answer to the cosmopolitanism versus endemism question is missing, partially because we still lack a definitive, rigorous, and operational species definition

applicable to protists. Species concepts vary greatly throughout the kingdoms of life and between protistan taxa, particularly classes. So in some classes of protists morphological species concepts dominate (Kristiansen 2008); in others, a mixture of morphological and molecular concepts is widely applied (Škaloud et al. 2014, 2015); and only in a few groups a biological species concept is used (Amato et al. 2007). It is necessary to have a uniform species concept, because species is a basic unit of biodiversity (Wilson 1992). Species delimitation is a fundamental requirement for our understanding of ecosystems and biodiversity, which is necessary for effective decision making about conservation efforts. However, species are not unchanging entities and evolve over time. We are therefore surrounded by a plethora of species that vary based on their evolutionary ages (Fig. 4), which can make it extremely difficult to perform species identification and delimitation. To date, a wide range of species concepts have been proposed, many of which are associated with several definitions. For example, Wilkins (2002) listed 26 species concepts. Moreover, many of these concepts are incompatible in that they can lead to different conclusions concerning the boundaries and number of species (De Queiroz 2007).

Current formulations of ubiquity theory use morphology to define the taxonomic units under consideration (Fenchel and Finlay 2006). Despite the long and fruitful history of the morphological species concept in protistology, morphology alone has been deemed inadequate due to a limited number of characters and uniform appearance (Rindi et al. 2008, 2011), which lead to taxonomic uncertainties. It is therefore not surprising that sharp discrepancies between morphology-based classifications and molecular phylogenies have emerged in numerous algal groups. Several genera, apparently well-defined from a morphological point of view, were recovered as polyphyletic or paraphyletic in molecular phylogenies (e.g., *Chlorella* - Huss et al. 1999, *Dictyosphaerium* - Bock et al. 2011, *Pediastrum* - Buchheim et al. 2005, *Printzina* and *Trentepohlia* - Rindi et al. 2009, *Trebouxia* - Škaloud and Peksa 2010). Molecular studies typically reveal a large amount of species-level diversity hidden within particular nominal taxa, that is, protist species that have been discovered and described using traditional methods based on morphological criteria (e.g. Von der Heyden et al. 2004, Amato et al. 2007, Lilly et al. 2007, Kooistra et al. 2008, Evans et al. 2009). Vanelslander et al. (2009) mentioned the term ‘‘cryptic diversity’’ referred to the existence of species that are morphologically indistinguishable, but genetically distinct. In many cases, the results of DNA sequence analyses initiated more detailed morphological studies, which often revealed subtle morphological differences between species, which are then called ‘‘pseudocryptic species’’ (Mann and Evans 2007). But Fenchel and Finlay (2006)

postulated that the use of genetic data brought confusion into the estimations of real diversity in protists.

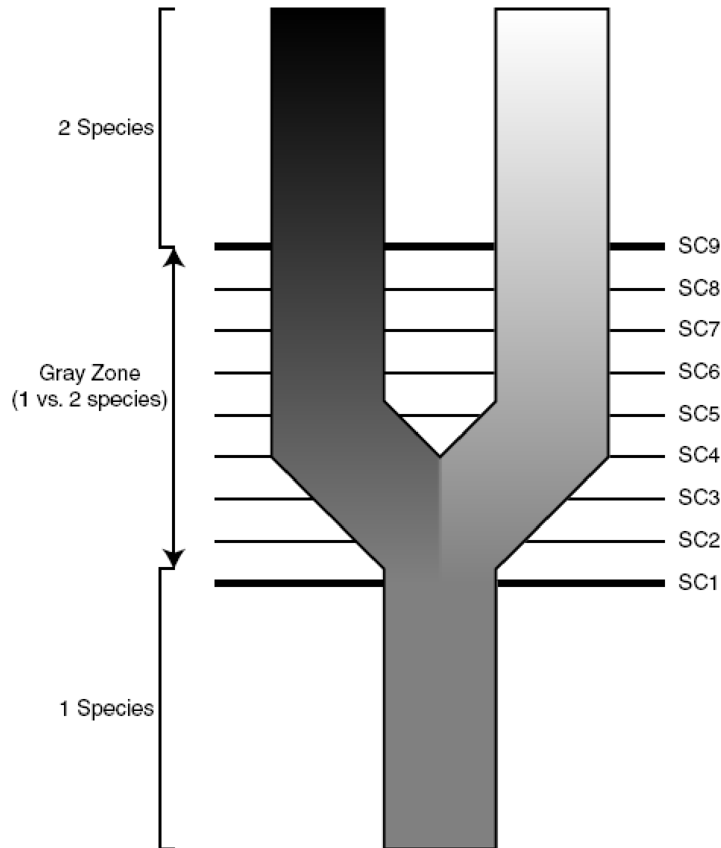

Figure 4. Lineage separation and divergence (speciation) and species concepts (after De Queiroz 2005). This highly simplified diagram represents a single lineage (species) splitting to form two lineages (species). The gradations in shades of grey represent the daughter lineages diverging through time, and the horizontal lines labelled SC (species criterion) 1 to 9 represent the times at which they acquire different properties (i.e., when they become phenetically distinguishable, diagnosable, reciprocally monophyletic, reproductively incompatible, ecologically distinct, etc.). The entire set of properties forms a grey zone within which alternative species concepts come into conflict. On either side of the grey zone, there will be unanimous agreement about the number of species. Before the acquisition of the first property, everyone will agree that there is a single species, and after the acquisition of the last property, everyone will agree that there are two. In between, however, there will be disagreement. The reason is that different contemporary species concepts adopt different properties (represented by the horizontal lines) as their species criteria-that is, as their cutoffs for considering a separately evolving lineage to have become a species. From De Queiroz (2007).

They proposed that the variation in molecular markers reflects the accumulation of neutral mutations over historical time, rather than the existence of morphologically indiscernible,

cryptic species. Complicated morphological identification has been demonstrated for some species, due to a high morphological plasticity (Rindi and Guiry 2002, Luo et al. 2006, Škaloud 2006, Darienko et al. 2010), and generally very high levels of morphological conservation and convergent evolution (Huss et al. 1999, Lilly et al. 2007, Krienitz and Bock 2012, Fučiková et al. 2014, Krienitz et al. 2015).

In the last decade, molecular methods usually led to the description of a hidden diversity, but did not resolve a problem with species delimitation. Consequently, numerous attempts have been done to define species algorithmically and many new methods for delimitation of species based on DNA sequences have been proposed (Puillandre et al. 2012, Carstens et al. 2013, Leavit et al. 2015). These methods appear to have a considerable potential, and their strengths and limitations have been extensively discussed, for example the generalized mixed Yule-coalescent model GMYC (Pons et al. 2006), PTP models (Zhang et al. 2013), the compensatory base changes (CBCs) in the ITS-2 rDNA molecule (Wolf et al. 2005). Several models use a Bayesian modelling approach to generate the posterior probabilities of species assignments taking into account the uncertainties due to unknown gene trees and the ancestral coalescent process (Yang and Rannala 2010), including the bPTP (Zhanget al. 2013), BAPS (Corander et al. 2008), STRUCTURAMA (Huelsenbeck et al. 2011), BP&P (Yang and Rannala 2010), and STACEY (Jones 2014). Some of these methods have been applied in recent studies on green microalgae (e.g., Sadowska-Des et al. 2014, Darienko et al. 2015, Malavasi et al. 2016). So far, however, their use has been limited to a few taxa and the applicability of their assumptions to wider range of groups of microchlorophytes needs to be verified with additional work.

In addition, an important practice strictly associated with species circumscription is the establishment of a link between molecular lineages and type specimens, which is necessary for the correct application of Linnean names. In general this problem does not exist for species of recent description: almost all species of green algae discovered in the last years were described using both morphological and molecular data, therefore DNA sequences produced from the type strains provide the reference data that establish the link between type material and molecular lineages. But problems arise with protists that were originally described using only morphological features (*e.g.*, see Ettl and Gärtner 1995). For these, apart for a few lucky exceptions (*e.g.*, Huss et al. 1999, Moniz et al. 2012), molecular data from type strains or specimens are not available. The morphology of many of these algae offers a limited set of morphological characters useful for identification and it is now well known that some of these characters are influenced by considerable phenotypic plasticity. A major

additional complication is the fact that some species were described by early botanists (*e.g.*, Linnaeus 1753, Agardh 1824, Kützing 1849, Nägeli 1849) who provided very brief descriptions lacking details about some morphological characters considered nowadays important. The consequence of these problems is that for many green microalgae the nature of the type specimen is ambiguous and to establish a link between type material and strains existing in nature is impossible, with consequent difficulties for the correct application of Linnean binomials.

Finally, the best way for protistan species delimitation is currently based on a combination of morphology, DNA sequences, physiology and ecology. Such polyphasic approaches seem to be promising for identification and circumscription of various species and genera (Schlegel and Meisterfeld 2003, Coesel and Krienitz 2008, Darienko et al. 2010, Malavasi et al. 2016).

1.4.Ecology

The complete statement of the ubiquity hypothesis for microorganisms is that 'everything is everywhere, but the environment selects' (Baas Becking 1934, De Wit and Bouvier 2006). Thus, knowledge of the ecological niche for these organisms ('the environment selects' part of the hypotheses) is needed in order to define their distribution, and niche-based models are used for predicting biogeographic distributions. But this is far from the reality, because the identification of ecological needs for microorganisms may not be as straightforward as for larger organisms. This is partly due to technical difficulties in measuring environmental parameters at the microscopic scale, but also because the presence of microorganisms in an area does not mean that the environment is suitable. Genetic theory predicts that the potential to adapt decreases linearly with decreasing effective population size (Hill 1982). This suggests that free-living protists, which often have huge population sizes, should have a high potential for local adaptive evolution similar to plants with large populations (Leimu and Fischer 2008). Large genetic diversity stored in these huge microbial populations could prevent the emergence of phenotypic plasticity. Nevertheless, the habitats of free-living protists may favour phenotypic plasticity. Free-living protists are found in all types of habitats, and some of those, such as lakes, have high environmental variability (both seasonally and spatially). This high environmental heterogeneity may favour adaptive phenotypic plasticity, and thus counteract local adaptation. In summary, the interplay of

external forces (e.g. environmental variability) as well as organismal and population characteristics (e.g. genomic variation, gene flow) should define whether protists tune to their environments by local adaptation or phenotypic plasticity (Rengefors et al. 2015).

Recently, a handful of studies have explored this topic and provide the evidence of high phenotypic plasticity (Finlay et al. 2006, Krenek et al. 2012, Rengefors et al. 2015), as well as of local adaptation (Huss et al. 2002, Boenigk et al. 2007, Weisse et al. 2011, Škaloud and Rindi 2013, Rengefors et al. 2015). For example, Rindi and Guiry (2002) reported that *Trentepohlia* and *Printzina* species formed perennial populations on different substrates in urban habitats in western Ireland. Similar ecological differentiation was reported for *Prasiola* (Trebouxiophyceae) (Moniz et al. 2012). Peksa and Škaloud (2011), who showed that specific lineages within the lichen photobiont genus *Asterochloris* exhibited clear environmental preferences, further demonstrated the influence of environmental factors. Huss et al. (2002) proposed that different *Chlorella*-like microalgae strains have independently adapted to extreme environments.

1.5.The genus *Klebsormidium* as a model organism

The genus *Klebsormidium* P. C. Silva, Mattox & W. H. Blackwell (Silva et al. 1972) is one of the most abundant microautotrophs in various terrestrial habitats (Ettl and Gärtner 1995, Lokhorst 1996, John 2002, 2003). The name *Klebsormidium* was established by Silva et al. (1972) to resolve the complicated nomenclatural history of this group of algae, which had been subjected to the taxonomic confusion in early stages. This genus belongs to the family Klebsormidiaceae, order Klebsormidiales, in the Streptophyte lineage of the Viridiplantae (Leliaert et al. 2012). Members consist of uniseriate filaments formed by cells having a parietal chloroplast with a single pyrenoid (Fig. 5) and reproducing asexually by biflagellate spores and fragmentation (Ettl and Gärtner 1995, Lokhorst 1996, John 2002, 2003, Rindi et al. 2008).

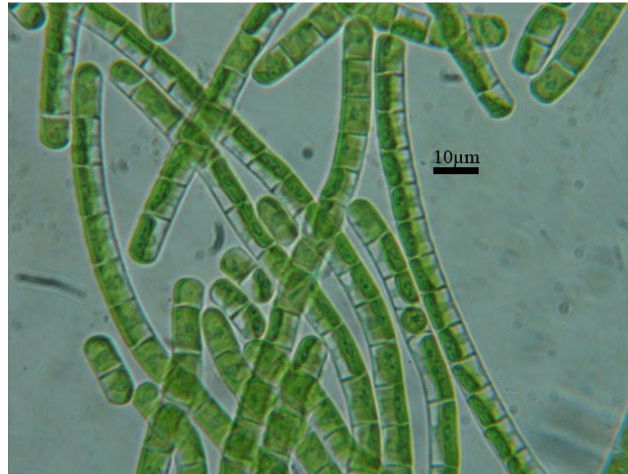

Figure 5. *Klebsormidium flaccidum*, morphology of vegetative filaments and cells. From Gaysiana et al. (2009).

The genus *Klebsormidium* currently includes 21 species distributed in a wide range of terrestrial and freshwater habitats (Ettl and Gärtner 1995, Lokhorst 1996). However, the species identification is very problematic due to a simple morphology with a few specific morphological characters, which are influenced by environmental conditions (Škaloud 2006). This led to the fact that although many cultured strains were identified as *Klebsormidium flaccidum*, phylogenetic analyses (Fig. 6) resolved that they in fact belong to the five distinct lineages (Rindi et al. 2011). Clarification of the taxonomic identity of *K. flaccidum* and *K. nitens*, the oldest taxa of the genus, has a critical importance for the classification of the Klebsormidiales, a group that occupies a distinct position in the Streptophyta (Lewis and McCourt 2004, Leliaert et al. 2012), and which is considered to be of great evolutionary and genomic interest (Civáň et al. 2014, Hori et al. 2014).

Klebsormidium has a widespread, cosmopolitan distribution, reported from polar to tropical regions (Ramanathan 1964, Lee and Wee 1982, Broady 1996, Lokhorst 1996, John 2002, 2003). The genus was predominantly studied in Europe (Rindi and Guiry 2004, Rindi et al. 2008, 2011, Škaloud and Rindi 2013, Mikhailyuk et al. 2015). Other areas, for example New Zealand (Novis 2006) and South Africa (Karsten et al. 2015), were studied to minimum extent, Similarly, its diversity in polar regions is largely unknown. In these areas, the majority of records comprise simple notes about their presence in various algal assemblages in both Antarctica (Mataloni et al. 2000, Cavacini 2001, Fermani et al. 2007) and the Arctic (Kaštovská et al. 2005, 2007, Stibal et al. 2006, Matula et al. 2007).

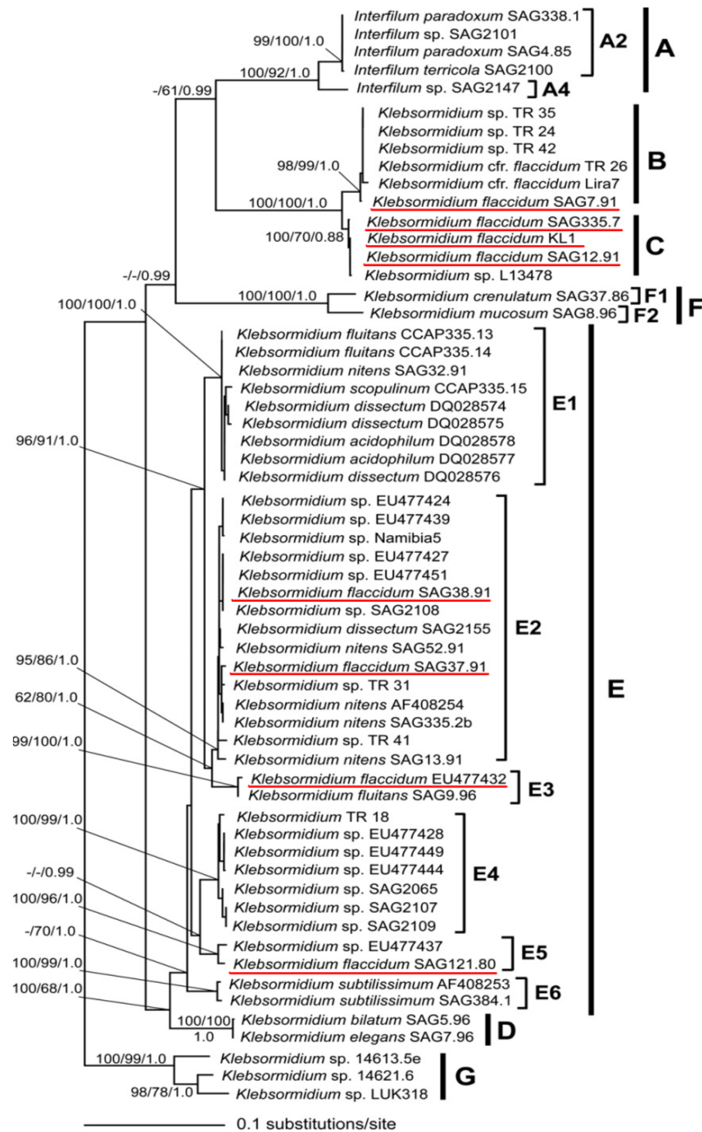

Figure 6. Strains morphologically determined as *Klebsormidium flaccidum* are highlighted by a red line. Phylogram inferred from Maximum-Likelihood analysis of the concatenated dataset *rbc*L-ITS rRNA in the Klebsormidiales, with bootstrap support (BS) and Bayesian Posterior Probabilities (PP) indicated at the nodes. From left to right and from top to bottom the support values correspond to Neighbor Joining BS, Maximum-Likelihood BS and Bayesian PP. BS values lower than 60% and PP lower than 0.8 are not reported. From Rindi et al. (2011).

Ecologically, the genus *Klebsormidium* is broadly distributed in various terrestrial and freshwater habitats, such as soil crusts (Lewis 2007, Mikhailyuk et al. 2015), tree bark (Handa et al. 1991, Nakano et al. 1991, Štifterová and Neustupa 2015), city walls and statues (Uher et al. 2005, Barberousse et al. 2006, Rindi et al. 2008), and rivers (Morison and Sheath 1985, John 2002, Lokhorst 1996, Novis 2006). Records of *Klebsormidium* in low pH habitats are available from many widely separated locations all over the world, mainly in waters (Novis 2006, Aguilera et al. 2010, Adlassnig et al. 2013) but also in soils (Lukešová and Hoffman

1996, Lukešová 2001, Lukešová and Hrčková 2011). The local distribution of *Klebsormidium* is generally influenced by different substrate preferences (Novis 2006, Rindi et al. 2008, 2011, Škaloud and Rindi 2013). Many strains of *Klebsormidium* show a wide range of adaptation to unfavourable conditions, such as prolonged desiccation (Karsten and Holzinger 2012, 2014, Herburger and Holzinger 2015, Karsten et al. 2015), low temperatures (Elster et al. 2008, Nagao et al. 2008), heavy metals (Gaysina et al. 2009) and UV radiation (Nagao et al. 2008, Kitzing et al. 2014).

2. Research objectives

In the present thesis, I tested the hypothesis "Everything is everywhere, but the environment selects" on a model organism, the genus *Klebsormidium*. I focused on both biogeography and ecology, and tried to find answers to the following questions.

Is there any biogeographical pattern in temperate zone and polar regions?

We demonstrated (paper 2) that a majority of genotypes represented by several isolates have a cosmopolitan distribution, whereas only a few genotypes were isolated in a single continent. Globally, the populations were genetically homogeneous; locally, the genotypes were patchily distributed. Whereas the population genetic investigation (paper 3) shows a clear evidence of a high dispersal capability of the superclade B, the absence of several genotypes in the polar regions points to the restricted distribution of the majority of *Klebsormidium* lineages.

Is there a comparable diversity in the polar and temperate regions?

Our investigations (paper 2) identified a large number of new, previously unrecognized lineages. A total of 44 genotypes were identified and more than 66% of these were reported for the first time. In paper 3, where we focused on polar regions, we detected a significant decrease of species richness towards the poles, i.e. the macroecological pattern typical for macroorganisms.

How can we connect species names with phylogenetic lineages?

In paper 5, we collected several specimens from the type localities of two species (*K. nitens* and *K. flaccidum*), for which we examined morphology in the field material and in culture, and obtained sequences of the *rbc*L gene. On the basis of the original descriptions, we concluded that the designation of the epitype of *K. flaccidum* was correct, whereas the epitype of *K. nitens* (which consists of a material collected tens of thousands of km apart from the type locality) was most probably incorrect. We discuss the implications of these decisions for the classification of *Klebsormidium* and, more generally, the importance of the correct choice of epitype material for the taxonomy of green microalgae.

How do the substrate and pH influence the diversity and speciation?

We determined (paper 4) a distinct ecophysiological differentiation among distantly and closely related lineages, thereby corroborating our hypothesis that the sympatric speciation of terrestrial algae is driven by ecological divergence. We clearly showed that pH is a critical ecological factor that influences the diversity of autotrophic protists in terrestrial habitats, and adaptation to low pH conditions has been developed multiple times independently in genus *Klebsormdium* (paper 1).

3. Original papers

3.1.Paper 1

Škaloud P., Lukešová A., Malavasi V., **Ryšánek D.**, Hrčková K. and Rindi F. (2014) Molecular evidence for the polyphyletic origin of low pH adaptation in the genus *Klebsormidium* (Klebsormidiophyceae, Streptophyta). *Plant Ecology and Evolution* 147: 333-345.

Molecular evidence for the polyphyletic origin of low pH adaptation in the genus *Klebsormidium* (Klebsormidiophyceae, Streptophyta)

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Background and aims – Algae living in low pH environments have been the subject of numerous studies, but their phylogenetic relationships with relatives found in non-acidic habitats are poorly known. In the present study we analyzed the morphology and phylogeny of acid-adapted strains of *Klebsormidium*, a genus of filamentous green algae frequently present in low pH environments.

Methods – Eighteen strains of *Klebsormidium* were collected from from low pH habitats in Europe and U.S.A., mainly from terrestrial sites affected directly or indirectly by carbon mining activities. These algae were isolated in culture and their phylogenetic relationships were studied using *rbc*L and ITS rDNA sequence data in a concatenated dataset.

Key results – In the molecular phylogeny the strains of *Klebsormidium* living in low pH habitats formed a polyphyletic assemblage. They were representative of sixteen lineages and corresponded morphologically to six species (*K. crenulatum*, *K. elegans*, *K. flaccidum*, *K. fluitans*, *K. nitens*, *K. scopulinum*), with the exception of four strains for which an unambiguous identification was not possible.

Conclusions – The genus *Klebsormidium* is a group of morphologically and physiologically dynamic algae in which the capacity of adaptation to low pH conditions has been developed multiple times independently. Extreme acidophilic populations probably originate from populations of various species growing locally when strongly acidic habitats become available. For the acid-adapted lineages of *Klebsormidium* examined here the current known distribution is geographically restricted, with the exception of a lineage containing strains from Czech Republic, New Zealand and Ohio.

Key words – Acid mine drainage, acidophilic algae, acidotolerant algae, extremophiles, Klebsormidiales, *Klebsormidium*, molecular systematics, phylogeny, Streptophyta, taxonomy.

INTRODUCTION

Sites characterized by low pH conditions occur both in terrestrial and aquatic environments throughout the world. In a few cases the high acidity is due to natural geochemical and biological features of the areas in which these sites are located and is not caused by anthropogenic influences. Some well-known cases are represented by certain sites affected by volcanic activities (Baffico et al. 2004, Pollio et al. 2005), streams with naturally low pH produced by leaching of fumic and fluvic acids from podocarp rainforests (Novis & Harding 2007) and rivers originating from areas with massive bodies of iron and copper sulfites (Aguilera et al. 2007a, 2007b). More often, however, the low pH is the result of human influence, mostly in the form of acid mine drainage released by mining activities (Lukešová 2001, Sabater et al. 2003, Novis & Harding 2007). The extent of these effects is usually localized, and consequently the distribution of highly acidic habitats is patchy and fragmented (Gross 2000, Weisse et al. 2011). However, sites with low pH exist in all continents and the overall extent of these habitats on global scale is not negligible; for example, in the recent past it was considered that approximately 15,000 km of streams in the U.S. were affected by acid mine drainage (Gross 2000).

Highly acidic environments pose challenging conditions for algae and cyanobacteria not only due to the low pH, butalso because acidity is often combined with extreme levels of other parameters. High concentrations of heavy metals such as Fe, Cu, Pb, Al and Zn are often recorded in soils and waters with low pH (Gross 2000, Aguilera et al. 2007b, Novis & Harding 2007, Spijkerman & Weithoff 2012), whereas at sites with geothermal activities acidity is accompanied by high temperatures (up to 83°C; Huss et al. 2002, Pollio et al. 2005, Spijkerman & Weithoff 2012). Additionally, most extremely acidic environments contain relatively low concentrations of dissolved organic carbon, and may therefore be considered oligotrophic (Johnson 1998), with further limitation for the growth of autotrophic organisms.

Due to such hostile conditions, the diversity of algae living in acidic environments is generally low (Huss et al. 2002, Sabater et al. 2003, Nancucheo & Johnson 2012, Spijkerman & Weithoff 2012), although some studies based on extended seasonal sampling and incorporating molecular data have revealed an unsuspected microbial eukaryotic diversity (Aguilera et al. 2006, 2007b). Organisms living in these environments can be separated into acidophiles (acidloving organisms, adapted to pH values as low as 0.05 and unable to grow at neutral pH) and acidotolerant (acid-tolerating organisms, with growth optima at higher pH but able to tolerate low values) (Johnson 1998, Gross 2000). Some algal taxa are particularly able to adapt to low pH and are a recurrent presence in acidic habitats; these are mainly unicellular

organisms such as species of the green algal genus *Chlamydomonas*, the euglenophyte *Euglena*, the chrysophyte *Ochromonas*, the diatom *Pinnularia* and red algae of the class Cyanidiophyceae (Johnson 1998, Huss et al. 2002, Ciniglia et al. 2004, Pollio et al. 2005, Novis & Harding 2007, Spijkerman & Weithoff 2012). However, some multicellular algae with filamentous habit may also occur in these habitats; of these, species of *Klebsormidium* P.C.Silva, Mattox & Blackwell are the most frequently recorded. Members of this genus belong to the streptophyte lineage of the Viridiplantae (Leliaert et al. 2012) and consist of uniseriate filaments formed by cells having a parietal chloroplast with a single pyrenoid and reproducing asexually by biflagellate spores (Lokhorst 1996, John 2002). *Klebsormidium* is one of the most widespread genera of green algae in the world, spanning in distribution from polar to tropical regions and occurring in a wide range of terrestrial and freshwater habitats (Lokhorst 1996, Rindi et al. 2008, Škaloud & Rindi 2013). Records of *Klebsormidium* in low pH habitats are available from many, widely separated locations all over the world, mainly in waters (Douglas et al. 1998, Stevens et al. 2001, Verb & Vis 2001, Brown & Wolfe 2006, Novis 2006, Valente & Gomes 2007, Bray et al. 2008, Lear et al. 2009, Urrea-Clos & Sabater 2009, Aguilera et al. 2010, Baffico 2010, Adlassnig et al. 2013) but also in soils (Lukešová & Hoffman 1996, Lukešová 2001, Lukešová & Hrčková 2011). Strains collected in low pH habitats are usually identified as *Klebsormidium rivulare* (Kütz.) M.O.Morison & Sheath (Morison & Sheath 1985, Stevens et al. 2001, Verb & Vis 2001), *Klebsormidium flaccidum* (Kütz.) P.C.Silva, Mattox & Blackwell (Lukešová & Hoffman 1996, Lukešová 2001, Sabater et al. 2003) and *Klebsormidium nitens* (Meneghini) Lokhorst (Lukešová 2001). Novis (2006) described *Klebsormidium acidophilum* Novis based on collections made in low pH streams in New Zealand. However, a taxonomic assessment of *Klebsormidium* at species level is hampered by several unresolved issues and the precise identity of several species, including the type species *K. flaccidum*, remains uncertain(Novis 2006, Škaloud 2006, Rindi et al. 2008, 2011,Škaloud & Rindi 2013). Currently, the molecular data available for strains of *Klebsormidium* from low pH habitats are limited, which is a major impediment for an assessment of their phylogenetic position and taxonomic identity.

Few studies have investigated the phylogenetic relationships between algae adapted to acidic conditions and their pH-neutral congeners. Besides the study of Novis (2006) for *Klebsormidium acidophilum*, results available for coccoid trebouxiophytes (Huss et al. 2002, Juárez et al. 2011) suggest that adaptation to low pH has taken place independently in different lineages and that in green microalgae acidophilic forms coexist with closely related neutrophilic forms. Here we investigate the phylogenetic relationships of low pH

Klebsormidium using *rbc*L and ITS sequences of several strains isolated from acidic soils and rivers in Europe and U.S.A. Using the phylogenetic framework built by previous molecular studies (Novis 2006, Mikhailyuk et al. 2008, Rindi et al. 2008, 2011, Škaloud & Rindi 2013), our goal is to clarify whether adaptation to low pH in *Klebsormidium* is monophyletic or not and in which known lineages of this genus this trait occurs. The results have important implications both in terms of speciation patterns and from a biogeographic point of view.

MATERIALS AND METHODS

Origin and isolation of *Klebsormidium* strains used in the study

Eighteen strains of *Klebsormidium* were obtained from low pH terrestrial and aquatic habitats as detailed in table 1. The strains were identified based both on morphological features (Printz 1964, Ettl & Gärtner 1995, Lokhorst 1996) and molecular data from recent studies (Novis 2006, Mikhailyuk et al. 2008, Novis & Visnovsky 2011, Rindi et al. 2011, Škaloud & Rindi 2013). With the only exception of the strain from Ohio, all strains were isolated in unialgal cultures and DNA extractions were performed on cultured material. The two strains from Sardinia, Italy (SCCA009 and SCCA011) were isolated using WARIS-H culture medium without soil extract (McFadden & Melkonian 1986). Stock cultures were established and maintained axenically at 25°C, 12:12 h L:D, under cool white luminescent light (80-100 µmol photons m^{-2} s^{-1}) in the Sardinian Culture Collection of Algae (SCCA), Interdepartmental Center of Environmental Science and Engineering (CINSA), University of Cagliari. The strains from Czech Republic and Germany were isolated in unialgal cultures using BBM culture medium (Andersen et al. 2005) and grown at 20–22°C, 18:6 h L:D, under cool white luminescent light (40–60 µmol photons m^{-2} s^{-1}). Stock cultures are maintained at 15°C under continuous light below 10 µmol photons m^{-2} s^{-1} in the Culture Collection of Soil Algae and Cyanobacteria at the Institute of Soil Biology, Academy of Sciences of the Czech Republic, České Budějovice.

DNA extraction, PCR and DNA sequencing

Total genomic DNA was extracted from the fresh cultures or silica-dried material using the Invisorb® Spin Plant Mini Kit (Invitek, Berlin, Germany). Algal DNA was resuspended in sterile dH2O and amplified by polymerase chain reaction (PCR). The ITS1-5.8S-ITS2 rDNA region was amplified using the primers Klebs-ITS-F (5’-GGA AGG AGA AGT CGT AAC AAG G-3’; Škaloud & Rindi 2013) and Klebs-ITS-R (5’-TCC TCC GCT TAG TAA TAT GC-3’; Škaloud & Rindi 2013). The *rbc*L gene was amplified using the

primers *rbc*L-KF2 (5'-ACT TAC TAC ACT CCT GAT TAT GA-3'; Škaloud & Rindi 2013) and *rbc*L-KR2 (5'-GGT TGC CTT CGC GAG CTA-3'; Škaloud & Rindi 2013) or the primers *rbc*L-KF376 (5'-TCA AAA CTT TCC AAG GTC CTC-3'; Rindi et al. 2008) and *rbc*L-KR2. All PCR reactions were performed in 20 μl reaction volumes, using the conditions described in Škaloud & Rindi (2013). The purified amplification products were sequenced with the PCR primers using an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730xl) at Macrogen Corp. in Seoul, Korea. Sequencing reads were assembled and edited using the SeqAssem programme (Hepperle 2004).

Sequence alignment and model selection

Multiple alignments of the newly determined ITS1 rDNA, ITS2 rDNA and *rbc*L sequences and other sequences selected from the DDBJ/EMBL/GenBank databases (electronic appendix 1) were manually built using MEGA 4 (Kumar et al. 2008), and then optimized using MAFFT, version 6, applying the Q-INS-i strategy (Katoh et al. 2002). The concatenated data matrix of unique sequences was 1,672 bp long and was 100% filled by the *rbc*L data (1,101 bp) and 79% filled by the ITS rDNA data (571 bp). The concatenated alignment used for the phylogenetic analyses consisted of 84 sequences. Sequences retrieved from GenBank were selected in order to represent all lineages currently known in the phylogeny of the Klebsormidiales (based primarily on Rindi et al. 2011); whenever possible, we used strains for which both *rbc*L and ITS sequence data were available. The appropriate substitution models for the ITS1 rDNA and ITS2 rDNA datasets and individual *rbc*L codon positions were selected using jModelTest 2.1.4 (Darriba et al. 2012). This BIC-based model selection procedure selected the following models: (1) TIM2ef + Γ for internal transcribed spacer ITS1, (2) K80 + Γ for internal transcribed spacer ITS2, (3), TIM1 + I for the first codon position of the *rbc*L gene, (4) JC + I for the second codon position of the *rbc*L gene, and (5) TRN + I + Γ for the third codon position of the *rbc*L gene.

Phylogenetic analyses

The phylogenetic tree was inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist et al. 2012). The analysis was carried out on a partitioned dataset using the different substitution models selected by jModelTest 2.1.4. The general structure of each substitution model was determined by the 'lset' command, and the model parameters were set using the priors defining the frequencies of nucleotides (statefreqpr) and nucleotide substitution rates (revmatpr) using the Dirichlet distribution. All parameters were unlinked

among partitions. Two parallel MCMC runs were carried out for five million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value between simultaneous runs was 0.00332. Finally, the burn-in was determined using the 'sump' command. Variance around the parameter estimates were verified in order to ensure that they were effectively modelled. Bootstrap analyses were performed by maximum likelihood (ML) and weighted parsimony (wMP) criteria using GARLI, version 0.951 (Zwickl 2006) and PAUP*, version 4.0b10 (Swofford 2002), respectively. ML analyses consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100,000). The wMP bootstrapping (1,000 pseudo-replicates) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences, and gap characters treated as missing data.

RESULTS

The strains examined in this study were collected mostly from terrestrial habitats, primarily soils in areas affected directly or indirectly by carbon mining activities. Although at several of these sites carbon mining had ceased a long time before our surveys and the sites were subsequently subjected to a natural succession, the pH measured at the time of collection was still fairly acidic, ranging between 2.0 and 5.0 (table 1). The only algae obtained from aquatic habitats were the two strains from Sardinia (SCCA009 and SCCA011, Malavasi 2012), which were growing in water bodies with very different characteristics (the Rio Irvi, where SCCA011 was collected, is a river affected by mining drainage with extremely high levels of heavy metals, where the pH of the water was 3.22; the strain SCCA009 was isolated from a large temporary pool with pH close to neutral).

Our analyses revealed a great morphological and phylogenetic diversity of the strains of *Klebsormidium* examined (figs 1, 2 & 3). Combining morphological observations andmolecular data, they were referred to six species (*K. crenulatum* (Kütz.) Lokhorst (fig. 2A), *K. elegans* Lokhorst (fig. 2B), *K. flaccidum* (fig. 2C–E), *K. fluitans* (F.Gay) Lokhorst (fig. 2F), *K. nitens* (fig. 3A–B), *K. scopulinum* (Hazen) H.Ettl & G.Gärtner), with the exception of four strains for which an unambiguous identification was not possible (fig. 3C–D). Details of the morphology of the strains are reported in table 2. In the concatenated ITS1-ITS2-*rbc*L phylogeny, the low pH strains of *Klebsormidium* formed a polyphyletic

assemblage (fig. 1). Overall, they were representative of sixteen lineages, which were separated in seven different clades belonging to four superclades delineated in recent studies (superclades D, E, F and G and clades E1, E2, E3, E4 as in Rindi et al. 2011 and Škaloud & Rindi 2013) (fig. 1). Some of these evolutionary units corresponded to well-circumscribed morphological species and the strains from low pH habitats belonging to these units were in good morphological agreement with them: this was the case for the superclade D (corresponding to *K. elegans*, fig. 2B), the superclade F (corresponding to *K. crenulatum*, fig. 2A) and the clade E3 (corresponding to *K. fluitans*, fig. 2F). Two unidentified strains (*Klebsormidium* sp. LUK S12 and LUK S66) belonged to the superclade G, a group discovered in recent molecular investigations (Rindi et al. 2011) and not yet resolved taxonomically; these strains displayed a key character typical of this superclade, the lobed chloroplast with a median incision (fig. 3D). Low pH strains identified morphologically as *K. flaccidum* belonged to the superclade E but did not form a monophyletic group and were scattered in three separate clades (E1, E2, E4). The clade E2 included the two aquatic strains from Sardinia (SCCA009 and SCC011); although collected from habitats with different pH conditions, they were closely related and their *rbc*L sequences differed by only a single nucleotide substitution. The two strains identified as *K. nitens* were not closely related and were recovered in separate clades (E2 and E4). The strain isolated from Ohio (PRC 2378) was identified as *K. scopulinum* and differed strikingly from all other strains for its markedly thin and long cells (4.5–5.5 μm wide, up to 10 times as long as wide). Such morphological difference was accompanied by a sharp phylogenetic separation: in the molecular phylogeny PRC 2378 was clearly separated from all other strains from low pH habitats sequenced in this study. However, it was recovered with high support in a group including other acid-tolerant *Klebsormidium* (*K. acidophilum* from New Zealand sequenced by Novis 2006); it was also in close relationship with the only strain of *K. scopulinum* for which *rbc*L and ITS sequence data were available (culture CCAP 335.15 isolated from the river Gannel, England).

Among our strains, only three had sequences identical to records already available in GenBank: *Klebsormidium* sp. LUK S12, *K. flaccidum* LUK S46 and *K. nitens* LUK S67. Therefore, most of the strains sequenced in this study appeared to be new lineages not detected in previous molecular studies, and some of them were remarkably distinct. *Klebsormidium* sp. LUK S50, in particular, was robustly placed in the superclade E but did not belong to any of the six clades delineated in this group in previous studies; it was recovered with high support as sister taxon to the clade E4. Two strains identified as *K. flaccidum* (LUK S01 and LUK S19) had identical *rbc*L and ITS sequences; they belonged to

the clade E1 but were distinct within it, representing the sister taxon to a clade formed by all other strains in this group.

Finally, it is worthy to note that none of the low pH *Klebsormidium* strains examined in this study and by Novis (2006) belonged to the superclades A, B and C. These groups include the genus *Interfilum* (superclade A), unidentified strains of *Klebsormidium* from natural habitats in eastern Europe (superclade B) and several strains of *K. flaccidum* deposited in culture collections (superclade C).

DISCUSSION

Our results clearly demonstrate that the capacity to adapt to low pH in *Klebsormidium* is phylogenetically widespread and does not represent a synapomorphy characterizing one or a few lineages. In our phylogeny, acid-adapted strains are widely interespersed among congeners living in habitats with neutral conditions. This situation is in agreement with the results of other phylogenetic studies focusing on microalgae from low pH environments, such as unicellular trebouxiophytes (Huss et al. 2002, Juarez et al. 2011), chlamydomonads (Gerloff-Elias et al. 2005, Pollio et al. 2005) and diatoms (Ciniglia et al. 2007). The fact that widely unrelated taxa possess the physiological and biochemical attributes that allow adaptation to low pH indicates that the genetic makeup on which these attributes are based is phylogenetically widespread among green algae. In the case of *Klebsormidium* this is not surprising, considering that members of this genus are equipped to withstand a wide range of extreme conditions and can grow in very hostile environments; records of *Klebsormidium* are available from biotic crusts of hot deserts (Lewis & Lewis 2005), alpine soil crusts (Karsten et al. 2010), hydrothermal springs (Brown & Wolfe 2006), Antarctic rocks and sand (Elster et al. 2008) and bases of concrete walls in trafficked urban streets (Rindi & Guiry 2004). In this genus colonization and adaptation to low pH habitats seem to be frequent events, probably more frequent than in other green algal taxa. Based on the present study and the results of Novis (2006), at least sixteen lineages of *Klebsormidium* have adapted to life in acidic habitats; this is a higher number than for the other genera of green algae that have been investigated to date in this regard. The nature and extent of adaptation to low pH, however, differ in different lineages. Some acid-adapted *Klebsormidium* strains are closely related to strains from non-acidic habitats; some have in fact identical or very similar *rbc*L and ITS sequences to strains isolated from different environments, which is a strong indication of conspecificity. In these cases we are probably dealing with generalist species with large dispersal and wide pH tolerance, able to survive equally well in acidic and non-acidic

environments. A situation of this type was demonstrated in other algae commonly found in low pH environments. For example, *Parachlorella kessleri* (Fott & Nováková) Krienitz et al. isolated from a mesothermal acidic pond in Argentina (pH 2.5–2.8) corresponded morphologically and had almost identical rDNA sequences to strains of the same species isolated from other environments (Juarez et al. 2011). The diatom *Pinnularia obscura* Krasske is considered a textbook example of an extremely acidotolerant species with global dispersal; strains from thermoacidic springs in Italy examined using morphological, ecophysiological and molecular data showed complete identity with strains from freshwater environments (Ciniglia et al. 2007). Conversely, other strains of *Klebsormidium* occurring in low pH environments are likely to represent evolutionary lineages that have genuinely evolved and specialized for life in acidic conditions. This type of scenario has been reported for other green algae considered real acidophilic organisms specialized for life at low pH, such as strains of *Chlamydomonas* from thermal springs in Italy (Pollio et al. 2005) and acidic mining lakes in Germany (Gerloff-Elias et al. 2005) that grow optimally at pH values < 3. In our phylogeny, good candidates are strains that form distinct lineages without a clear sister species/taxon, such as *Klebsormidium* sp. LUK S50, *K. flaccidum* LUK S01/LUK S19 and *K. nitens* LUK S48. We believe that these strains originated based on the hypothesis that extreme acidophilic populations establish from populations of various species growing locally when strongly acidic habitats become available. Other studies conducted in different environments have concluded that extreme conditions apparently unfavourable for survival and growth of green microalgae drive the evolution of these organisms (e.g. extreme aridity of North American deserts, Lewis & Lewis 2005); it can be therefore expected that the same applies to strong acidity. This would also be consistent with the possibility suggested by Škaloud & Rindi (2013) that selective sweep combined with the selection of new mutants differing in ecological niche may have played a major role in the differentiation of *Klebsormidium*. Physiological studies determining the optimal pH and the limits of the pH range in which different strains can grow would be very useful to understand better the nature and extent of adaptation to low pH conditions. Unfortunately, however, physiological data on acid adaptation in *Klebsormidium* are restricted to the experiments of Novis (2006) on *K. acidophilum* and *K. dissectum* from New Zealand. This is somewhat surprising, considering that *Klebsormidium* has been reported frequently in low pH habitats and that other aspects of the physiologyof this genus have been studied with great detail in recent years (e.g. Elster et al. 2008, Nagao et al. 2008, Karsten et al. 2010, Karsten & Rindi 2010, Kaplan et al. 2012, Karsten & Holzinger 2012, Karsten et al. 2013). Novis (2006) found that both *K. acidophilum*

and *K. dissectum* were able to grow in a similar range of pH (approximately 2.0 to 9.0), but the healthiest filaments of *K. acidophilum* were observed at pH 2.4, whereas the healthiest filaments of *K. dissectum* were found at pH 4.8–6.2; combined with other morphological data, this supports the separation of *K. acidophilum* as a low pH species. Interestingly, other strains closely related to *K. acidophilum* examined in subsequent studies were collected from different geographical regions but also isolated from acidic environments: *Klebsormidium* sp. K44 from an acidic peat bog in the Czech Republic (Škaloud & Rindi 2013) and *K. scopulinum* PRC 2378 (sequenced in this study) from the acidic seep of an abandoned coal mine in Ohio.

The idea that acid-adapted strains of *Klebsormidium* originate independently from generalist populations when acidic habitats become available is also partially supported by their high biogeographical diversity. It is remarkable that almost each acid-adapted lineage discovered in this study was restricted to a single site or a few sites in the same geographical area. At present none of these lineages appear to have a wide geographical distribution, with the only major exception of the lineage containing *K. acidophilum* and *K. scopulinum* (which, as mentioned above, includes strains from Czech Republic, New Zealand and Ohio). Biogeography and dispersal of acidophilic organisms are fascinating but poorly understood topics, and have been investigated in detail only for few taxa. Some authors concluded that acidophilic species have worldwide distribution (Gimmler 2001, Gerloff-Elias et al. 2005), conforming essentially to the neutral model of ubiquitous dispersal of microorganisms (everything is everywhere, but the environment selects; Baas Becking 1934). However, molecular studies that have focused on some acid-adapted morphospecies have unraveled genetic heterogeneity correlated to geographical distribution; cyanidialean red algae belonging to the genus *Galdieria* are the best-know example (Pinto et al. 2003, Ciniglia et al. 2004). Not surprisingly, this pattern is found in algae that are obligate acidophiles restricted to thermal acidic sites. An acidic environment is essential for the survival of these organisms, that cannot establish subpopulations in non-acidic habitats (Gross et al. 2001); their inability to reach easily other sites with suitable characteristics determines geographic isolation, with consequent high genetic differentiation (Ciniglia et al. 2004). The patterns observed for *Klebsormidium* conform to some extent to this situation; this may be an indication that many low pH *Klebsormidium* strains have reached a high level of specialization to acidic habitats, and do not disperse easily. This possibility, however, requires confirmation based on physiological data and further molecular sampling from other geographic regions. At the moment the data available for low pH strains are geographically biased (most of our strains

are from central Europe) and sequence data of strains from other continents could reveal a different scenario.

The morphological identification of our strains based on characters traditionally used for species circumscription in *Klebsormidium* (Printz 1964, Ettl & Gärtner 1995, Lokhorst 1996, John 2002) was straightforward for most of our strains. Our results, however, confirm the discrepancies between molecular phylogeny and morphology-based taxonomy evidenced by previous molecular studies (Mikhailyuk et al. 2008, Rindi et al. 2008, Novis & Visnovsky 2011, Rindi et al. 2011). Some species, such as *Klebsormidium crenulatum* and *K. elegans*, can be linked unambiguously with molecular phylogenetic groups, and the low pH strains exhibitng the morphology of these species belong to the expected clades. Conversely, the morphology of the type species *K. flaccidum* is homoplasious and largely widespread in the phylogeny of the genus. This situation was already highlighted by previous studies (Rindi et al. 2011, Škaloud & Rindi 2013) and is confirmed here: low pH strains morphologically referable to *K. flaccidum* belong to three different clades. The taxonomic circumscription of this species remains an open problém which cannot be solved by sequencing the type specimen, due to its poor quality (a small sample formed by a few filaments embedded in a drop of mud). Since the morphology of this species is so widespead in different clades, selection of a specimen from the type locality (Strasbourg, France) would also not guarantee to obtain the alga actually used for the description of the species (Kützing 1849). Therefore, the designation of an epitype specimen based on a subjective choice appears to be the only feasible solution of the reassessment of this species (Rindi et al. 2011). To a lesser extent, the same considerations apply to *K. nitens*, which is also polyphyletic; in this case, however, there is a phylogenetic group which appears a good candidate to be linked with this morphospecies (the clade E2, in which most strains referred to this species are recovered; Rindi et al. 2011, Škaloud & Rindi 2013).

An interesting discovery of this study is the phylogenetic positioning of the strains LUK S12 and LUK S66 in the superclade G. The superclade G represents a lineage recently discovered, formed mainly by strains isolated from biotic crusts of subdesertic areas in Namibia and South Africa (Rindi et al. 2011). Within this superclade, the strains LUK S12 and LUK S66 form a well-supported lineage with *Klebsormidium* sp. LUK 318, another strain isolated from eastern Europe (discarded material on soil previously subjected to coal mining in the Czech Republic). The two strains sequenced here exhibited a four-lobed chloroplast with a median incision, a character that appears to be diagnostic for this group. It will be very interesting to verify if this group has a wider geographical distribution and if it also occurs in

different types of habitats; it can be expected, in particular, that strains of *Klebsormidium* from desertic areas in North America (Lewis & Fletchner 2002, Lewis & Lewis 2005) will turn out to belong to this superclade.

An additional point that requires some discussion is the high morphological plasticity related to environmental factors observed in *Klebsormidium*, an aspect that has complicated many morphological studies. It has been shown that the culture conditions and the age of cultures can significantly affect some morphological characters which were considered useful for species identification for a long time (Škaloud 2006, Rindi et al. 2008). The limited data available indicate that pH conditions may play a major role in this regard: Novis (2006) highlighted a strong effect of pH on the morphologies of *K. acidophilum* and *K. dissectum*, showing that characters such as cell shape, chloroplast shape and amount of granules deposited in the cytoplasm varied considerably in different pH conditions. Variations in pH are also known to affect cell shape in other green microalgae, mainly determining an overall reduction of the cell surface relative to the cell volume (Coesel 1982, Černá & Neustupa 2010). This phenomenon is considered a functional response aimed at reducing osmotic stress. Algae living in low pH habitats are able to maintain a fairly constant, neutral, cytosolic pH over a wide range of external pH values (Gerloff-Elias et al. 2005, Bethmann & Schönknecht 2009); the maintenance of a neutral cytosolic pH under low pH conditions is an energy-demanding process that involves considerable metabolic costs, and a reduced cell surface contributes to reducing these costs (Černá & Neustupa 2010). Due to lack of experimental data, we are not able to demonstrate similar effects in our *Klebsormidium* strains and to assess their morphological variation in different pH conditions; we expect, however, that variations similar to those reported by Novis (2006) are probably generalphenomenon.

In conclusion, the genus *Klebsormidium* is a morphologically and physiologically dynamic algal group in which the capacity of adaptation to low pH conditions has been developer multiple times independently. Further studies aimed at clarifying the extent of this adaptation and the molecular features that determine it have the potential to shed light into many fascinating aspects of the biology of acidophilic and acidotolerant organisms that are still poorly understood.

ACKNOWLEDGEMENTS

We express our best wishes to our colleague Pierre Compère for his 80th birthday and congratulate him for the achievements of his long and productive scientific career. We are very grateful to Sam Drerup and Morgan Vis for collecting and sending the strain of

Klebsormidium scopulinum from Ohio. P. Š. acknowledges financial support from Charles University Prague. V. M. gratefully acknowledges financial support from the Sardinia Regional Government (Italy) through the project "Master and Back – Percorsi di rientro" (P.O.R. Sardegna F.S.E. 2007–2013). The financial support of Research Unit of the National Interuniversity Consortium "The Chemistry for the Environment" and Interdepartmental Center of Environmental Science and Engineering (CINSA), University of Cagliari, is gratefully acknowledged. The work was also partly supported by "Fondazione Banco di Sardegna" (Sassari, Italy).

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TABLES

Table 1. Details of the strains of *Klebsormidium* used in the study.

Strain	Identification	Collection data	GenBank accession number (rbcL)	GenBank accession number (ITS)
LUK S01	*Klebsormidium flaccidum*	Sokolov coal mining area, Czech Republic. Coal clay, 30-years old forest, spontaneous succession (pH 3.05). Alena Lukešová, April 2008.	To be deposited	To be deposited
LUK S02	*Klebsormidium elegans*	Sokolov coal mining area, Stará Chodovská, Czech Republic. Coal clay, 40-years old forest, spontaneous succession (pH 3.4). Alena Lukešová, April 2008.	To be deposited	To be deposited
LUK S03	*Klebsormidium fluitans*	Sokolov coal mining area, Stará Chodovská, Czech Republic. Coal clay, 40-years old forest, spontaneous succession (pH 3.4). Alena Lukešová, April 2008.	To be deposited	To be deposited
LUK S06	*Klebsormidium crenulatum*	Giant Mountains, Vysoké Kolo Mount (1510 m a.s.l), Czech Republic. Polygonal soil on granite, biological crust (pH 4.0). Alena Lukešová, June 2006.	To be deposited	To be deposited
LUK S12	*Klebsormidium* sp.	Slavkovský les, peat bog Krásno, Czech Republic. Peat, meliorated area with dense cover dominated by *Calluna vulgaris*, thick litter layer (pH 4.0). Alena Lukešová, June 2007.	To be deposited	To be deposited
LUK S19	*Klebsormidium flaccidum*	Sokolov coal mining area, Czech Republic. Coal clay, 30-years old forest, spontaneous succession (pH 3.0). Alena Lukešová, April 2008.	To be deposited	To be deposited
LUK S29	*Klebsormidium crenulatum*	Vintířov, Sokolov coal mining area, Czech Republic. Volcanic ash mixed with coal clay, 10 years after mining, spontaneous succession (pH 3.45). Alena Lukešová, April 2008.	To be deposited	To be deposited
LUK S46	*Klebsormidium flaccidum*	Weissacker Berg, near Cottbus, Lusatian lignite mining area, Germany. Tertiary carboniferous and pyritic sand, ameliorated with fly ash, 2 years after mining (pH 2.9). Alena Lukešová, May 1997.	To be deposited	-
LUK S48	*Klebsormidium nitens*	Meuro, Lusatian lignite mining area, Germany. Sandy soil, 20-years old pine plantation (pH 5.0). Alena Lukešová, May 1997.	To be deposited	To be deposited
LUK S50	*Klebsormidium* sp.	Weissacker Berg, near Cottbus, Lusatian lignite mining	To be deposited	-

		area, Germany. Tertiary carboniferous and pyritic sand, ameliorated with fly ash and sewage sludge, 2 years after mining (pH 2.9). Alena Lukešová, May 1997.		
LUK S63	*Klebsormidium* sp.	Weissacker Berg, near Cottbus, Lusatian lignite mining area, Germany. Tertiary carboniferous and pyritic sand, 1 year after mining (pH 2.7). Alena Lukešová, October 1999.	To be deposited	-
LUK S64	*Klebsormidium flaccidum*	Weissacker Berg, near Cottbus, Lusatian lignite mining area, Germany. Tertiary carboniferous and pyritic sand, ameliorated with fly ash and compost, 4 yrs after mining (pH 2.9). Alena Lukešová, October 1997.	To be deposited	To be deposited
LUK S66	*Klebsormidium* sp.	Weissacker Berg, near Cottbus, Lusatian lignite mining area, Germany. Tertiary carboniferous and pyritic sand, ameliorated with fly ash and mineral fertilizers, 2 years after mining (pH 2.9). Alena Lukešová, May 1997.	To be deposited	To be deposited
LUK S67	*Klebsormidium nitens*	Domsdorf, Lusatian lignite mining area, Germany. Sandy soil, 34 years old pine plantation (pH 4.1). Alena Lukešová, October 1999.	To be deposited	To be deposited
LUK S68	*Klebsormidium elegans*	Domsdorf, Lusatian lignite mining area, Germany. Sandy soil, 34 years old pine plantation (pH 4.0). Alena Lukešová, May 1997.	To be deposited	To be deposited
PRC 2378	*Klebsormidium scopulinum*	Carbondale, Ohio, U.S.A. Acidic seep of an abandoned coal mine, heavily armored with iron oxide precipitation (pH < 4.0). Sam Drerup, 3 January 2014.	To be deposited	-
SCCA009	*Klebsormidium flaccidum*	Arquerì, forest of Montarbu, Seui, Ogliastra, central-eastern Sardinia, Italy. About 1000 m a.s.l. Temporary pool on side of untarred road (pH 7.2). Veronica Malavasi, 18 January 2009.	To be deposited	-
SCCA011	*Klebsormidium flaccidum*	Rio Irvi-Piscinas, area Montevecchio-Ingurtosu, southwestern Sardinia, Italy. 370 m a.s.l. Water of Rio Irvi (pH 3.22). Veronica Malavasi, 14 September 2012.	To be deposited	-

Table 2. Morphology of the strains of *Klebsormidium* used in the study.

Strain	Identification	Morphology
LUK S01	*Klebsormidium flaccidum*	Long filaments, without tendency to break, cells cylindrical, 6-8 µm wide, 7-11 µm long, 1-1.8 times as long as wide. Chloroplast covering about 70-80% of the cell, with smooth margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S02	*Klebsormidium elegans*	Short filaments, with tendency to break into short fragments. Occasionally, filaments were spirally coiled. Cells cylindrical, 9-13 µm wide, 6-12 µm long, 0.5-1.2 times as long as wide. Chloroplast covering 70% of the cell wall, with smooth margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S03	*Klebsormidium fluitans*	Long filaments, without tendency to break, cells cylindrical (subglobular), 6-8 µm wide, 5-9 µm long, 0.6-1.2 times as long as wide. Chloroplast covering about 50% of the cell, with smooth margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S06	*Klebsormidium crenulatum*	Long filaments, without tendency to break, cells cylindrical, 11-13 µm wide, 8-12 µm long, 0.6-1 times as long as wide. Chloroplast covering about 75% of the cell, with evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S12	*Klebsormidium* sp.	Short filaments, with tendency to break into very short fragments (in many cases 1-2 cells). Cell cylindrical, 5-6 µm wide, 9-12 µm long, 1.5-2.4 times as long as wide. Chloroplast covering about 35-50% of the cell, with unclear small pyrenoid. Fully developed chloroplasts often deeply incised midway. H-shaped pieces observed. Release of zoospore not observed.
LUK S19	*Klebsormidium flaccidum*	Long filaments, without tendency to break, cells cylindrical, 5.5-8 µm wide, 6-11 µm long, 0.75-1.8 times as long as wide. Chloroplast covering about 50-60% of the cell, with smooth margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S29	*Klebsormidium crenulatum*	Long filaments, without tendency to break, cells cylindrical, 10-15 µm wide, 8-15 µm long, 0.5-1.5 times as long as wide. Chloroplast covering about 50% of the cell, with smooth or grooved margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S46	*Klebsormidium flaccidum*	Short filaments, with tendency to break, cells cylindrical, 7-8 µm wide, 7-16 µm long, 0.9-2.2 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. H-shaped pieces not observed. Release of zoospore not observed.
LUK S48	*Klebsormidium nitens*	Short filaments, with tendency to break, cells cylindrical, 4-5 µm wide, 8-15 µm long, 1.6-3.8 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. H-shaped pieces not observed. Release of zoospore not observed.
LUK S50	*Klebsormidium* sp.	Long filaments, without tendency to break, cells cylindrical to subglobular, 9-11 µm wide, 8-11 µm long, 0.9-1.2 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S63	*Klebsormidium* sp.	Long filaments, without tendency to break, cells cylindrical, 8-9 µm wide, 8-18 µm long, 0.9-2.3 times as long as wide. Chloroplast covering about 50-70% of the cell, with evident pyrenoid. H-shaped pieces

		observed. Release of zoospore not observed.
LUK S64	*Klebsormidium flaccidum*	Short filaments, with tendency to break, cells cylindrical, 6-7 µm wide, 10-17 µm long, 1.4-2.8 times as long as wide. Chloroplast covering about 75% of the cell, without evident pyrenoid. H-shaped pieces not observed. Release of zoospore not observed.
LUK S66	*Klebsormidium* sp.	Short filaments, but without tendency to break, cells cylindrical, 6-7 µm wide, 8-13 µm long, 1.1-2.2 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. Fully developed chloroplasts occasionally incised midway. H-shaped pieces observed. Release of zoospore not observed.
LUK S67	*Klebsormidium nitens*	Short filaments, with tendency to break, cells cylindrical, 5-6 µm wide, 8-14 µm long, 1.3-2.8 times as long as wide. Chloroplast covering about 50% of the cell, without evident pyrenoid. H-shaped pieces not observed. Release of zoospore not observed.
LUK S68	*Klebsormidium elegans*	Long filaments, with tendency to break, cells cylindrical, but becoming doliiform with increasing age, 8-9 µm wide, 6-10 µm long, 0.7-1.3 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
PRC 2378	*Klebsormidium scopulinum*	Alga forming dense mats consisting of strong, dense filaments long up to 40 cm, without tendency to break. Cells cylindrical, 4.5-5.5 µm wide, 2-10 times as long as wide. Chloroplast extending for the whole length of the cell, covering about 50% of the cell wall, without evident pyrenoid. H-shaped pieces and constrictions between adjacent cells absent. Release of zoospores not observed.
SCCA009	*Klebsormidium flaccidum*	Alga with filamentous structure, forming long filaments with limited tendency to break. Filaments easily adhering to the substratum. Cells cylindrical, 6-7 µm wide, 0.5-3 times as long as wide (mainly about 1.5). A few intercalary cells are subglobular and distinctly larger. H-shaped pieces not observed. Constrictions between adjacent cells occasionally present, mainly in fragmenting portions of the alga. Chloroplast parietal, extending for the whole length of the cell and covering about 50% of the side wall, sometimes with irregular margins. Pyrenoid small, without a clear starch envelope. Release of zoospores not observed. In some parts the filaments are distinctly bent with elbow-like habit, suggesting that the germination pattern of the zoospores is bipolar.
SCCA011	*Klebsormidium flaccidum*	Filamentous alga, with limited tendency to break into short fragments. Cells cylindrical, 6-7.5 µm wide, 1-3 times as long as wide (mainly about 1.5). Several intercalary cells are subglobular and distinctly larger. H-shaped pieces not observed. Constrictions between adjacent cells occasionally present, mainly in fragmenting portions of the alga. Chloroplast parietal, extending for the whole length of the cell and covering about 50% of the side wall, sometimes with irregular margins. Pyrenoid small, without a clear starch envelope. Release of zoospores not observed. In some parts the filaments are distinctly bent with elbow-like habit, suggesting that the germination pattern of the zoospores is bipolar.

FIGURES

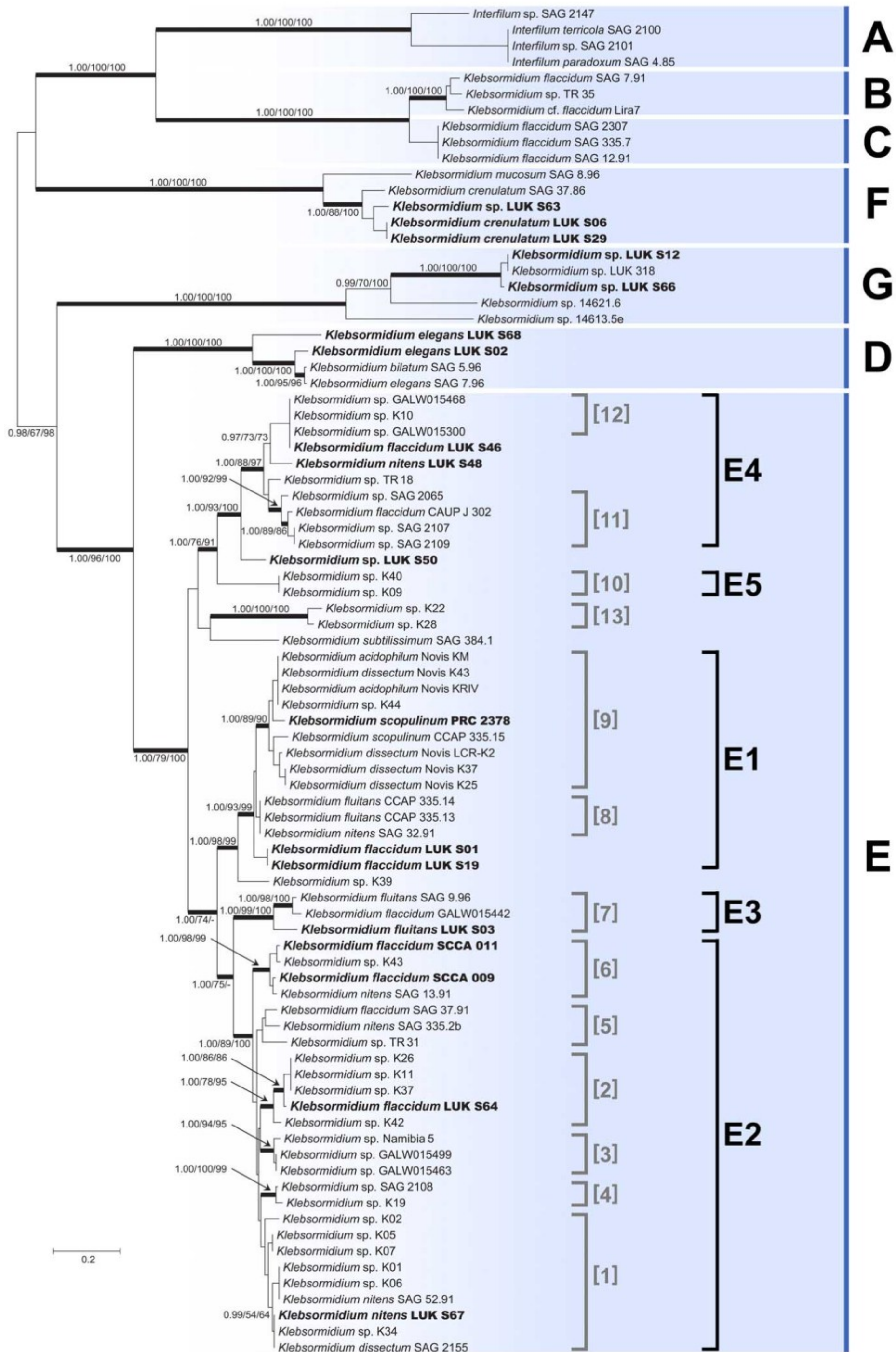

Figure 1. Phylogram obtained from Bayesian analysis based on the combined *rbc*L and ITS rDNA dataset, showing the position of investigated *Klebsormidium* strains and their relatives. Values at the nodes indicate statistical support estimated by MrBayes posterior node probability (left), maximum likelihood bootstrap (middle) and maximum parsimony bootstrap (right). Thick branches represent nodes receiving the highest PP support (1.00). Species affiliation to seven superclades (A–G) and five clades (E1-E5) sensu Rindi et al. (2011) is indicated, as well as the affiliation to 13 lineages sensu Škaloud & Rindi (2013). Scale bar indicates number of substitutions per site.

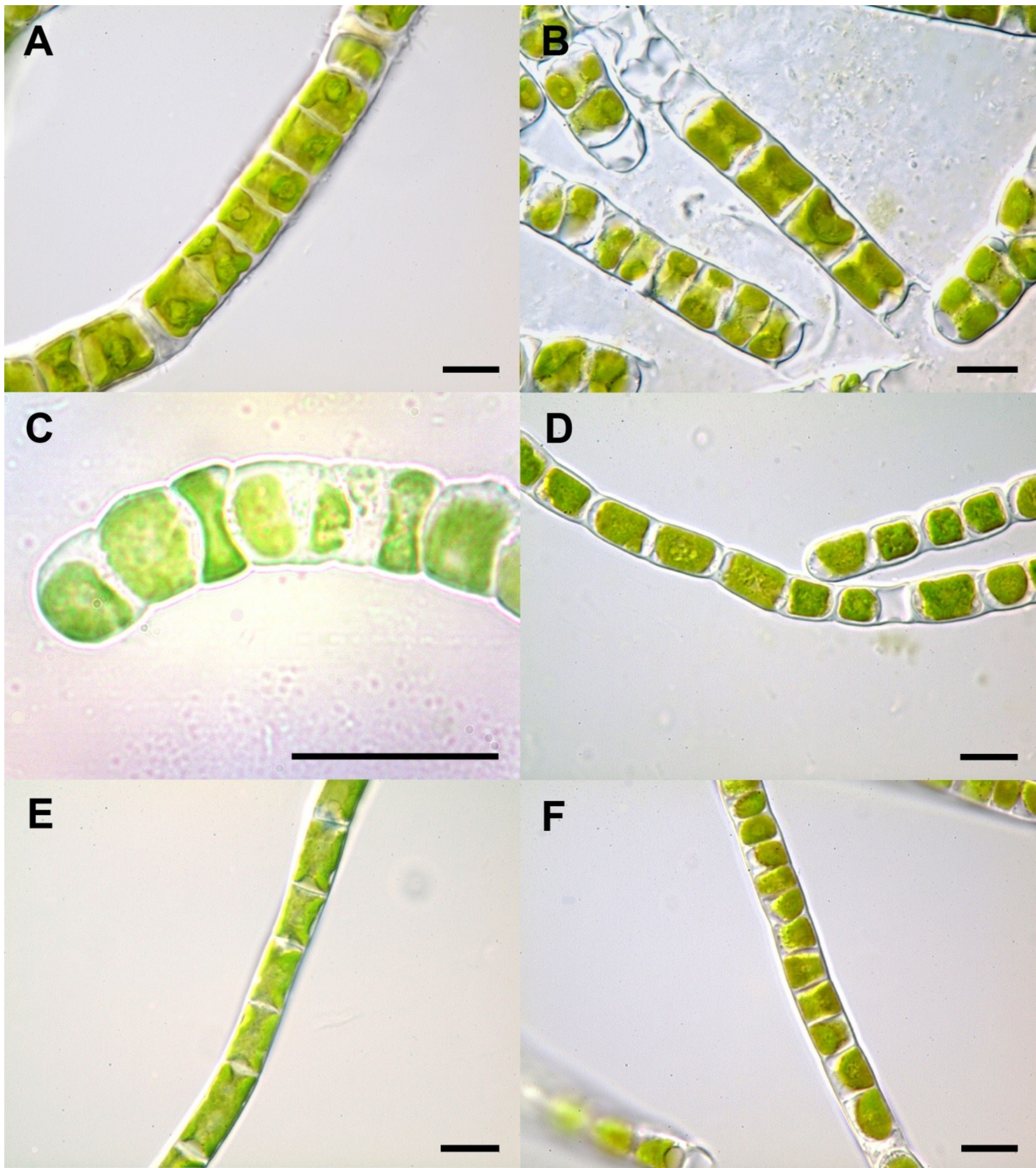

Figure 2. Morphology of strains of *Klebsormidium* from low pH habitats: A, *Klebsormidium crenulatum* S06; B, *Klebsormidium elegans* S02; C, *Klebsormidium flaccidum* SCCA011; D, *Klebsormidium flaccidum* S19; E, *Klebsormidium flaccidum* S64; F, *Klebsormidium fluitans* S03. Scale bars represent 10 µm.

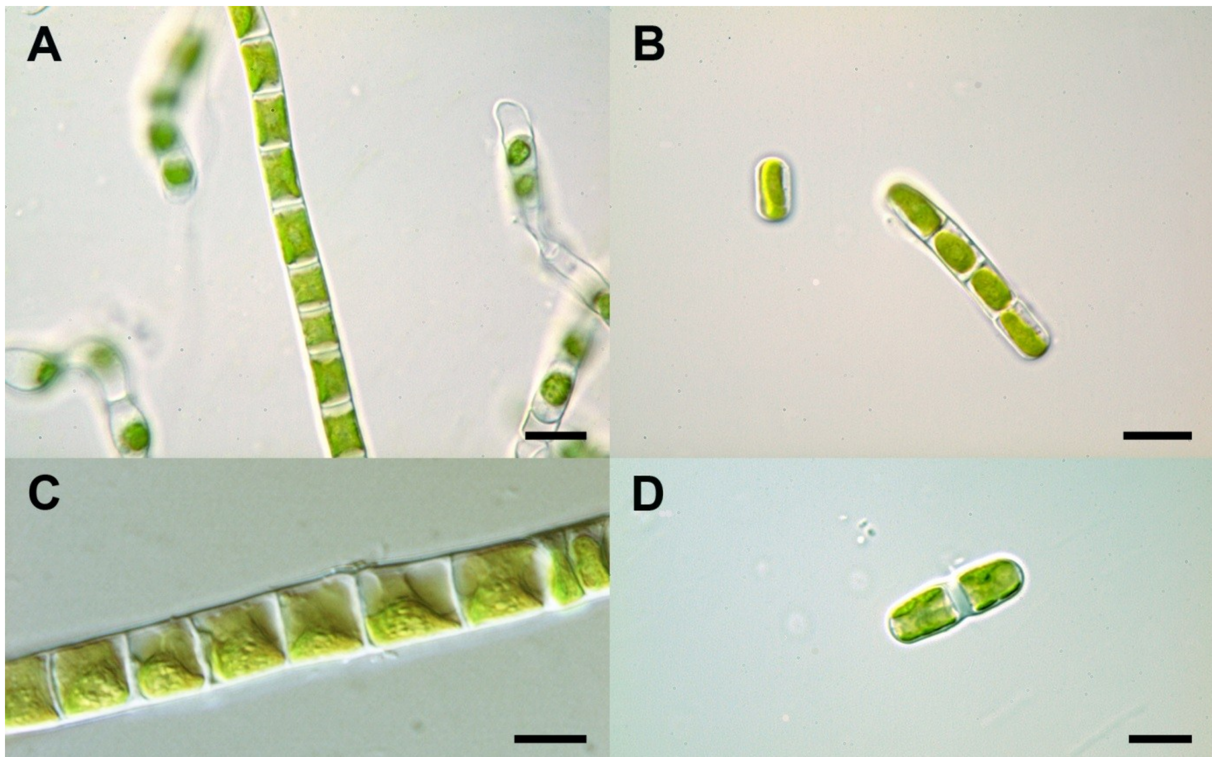

Figure 3. Morphology of strains of *Klebsormidium* from low pH habitats: A, *Klebsormidium nitens* S48; B, *Klebsormidium nitens* S67; C, *Klebsormidium* sp. S50; D, *Klebsormidium* sp. S12. Scale bars represent 10 µm.

SUPPLEMENTARY DATA

Table 1. Additional sequences of Klebsormidiales used for the phylogenetic analyses.

Identification	**GenBank accession number ITS1 / ITS2**	**GenBank accession number (*rbc*L)**	**References**
Interfilum paradoxum SAG 4.85	EU434016 / HQ654194	HQ613239	Mikhailyuk et al. 2008, Rindi et al. 2011
Interfilum terricola SAG2100	EU434040 / EU434040	HQ613238	Mikhailyuk et al. 2008, Rindi et al. 2011
Interfilum sp. SAG 2101	EU434035 / EU434035	HQ613236	Mikhailyuk et al. 2008, Rindi et al. 2011
Interfilum sp. SAG 2147	EU434039 / EU434039	HQ613237	Mikhailyuk et al. 2008, Rindi et al. 2011
Klebsormidium acidophilum Novis KM		DQ028577	Novis 2006
Klebsormidium acidophilum Novis KRIV		DQ028578	Novis 2006
Klebsormidium bilatum SAG 5.96	AM490841 / AM490841	EU477425	Rindi et al. 2008, Sluiman et al. 2008
Klebsormidium crenulatum SAG 37.86	HQ654128 / HQ654194	HQ613241	Rindi et al. 2011
Klebsormidium dissectum Novis LCR-K2		EF589144	Novis & Visnovsky 2011
Klebsormidium dissectum Novis K25		DQ028574	Novis 2006
Klebsormidium dissectum Novis K37		DQ028575	Novis 2006
Klebsormidium dissectum Novis K48		DQ028576	Novis 2006
Klebsormidium dissectum SAG 2155	EF372518 / EF372518	EU477429	Rindi et al. 2008, Sluiman et al. 2008
Klebsormidium elegans SAG 7.96	AM490840 / AM490840	EU477430	Rindi et al. 2008, Sluiman et al. 2008
Klebsormidium flaccidum CAUP J 302	HE649335 / HE649335	HE649364	Škaloud & Rindi 2013
Klebsormidium flaccidum GALW015442		EU477432	Rindi et al. 2008
Klebsormidium flaccidum SAG 7.91	EU434019 / EU434019	EU477435	Mikhailyuk et al. 2008, Rindi et al. 2008
Klebsormidium flaccidum SAG 12.91	EU434024 / EU434024	EU477436	Mikhailyuk et al. 2008, Rindi et al. 2008
Klebsormidium flaccidum SAG 37.91	HQ654131 / HQ654197	HQ613244	Rindi et al. 2011
Klebsormidium flaccidum SAG 335.7	HQ654137 / HQ654203	EU477434	Rindi et al. 2008, 2011
Klebsormidium flaccidum SAG 2307	AM490838 / AM490838	HQ613242	Sluiman et al. 2008, Rindi et al. 2011
Klebsormidium cf. flaccidum Lira7	HQ654139 / HQ654205	HQ613243	Rindi et al. 2011

Klebsormidium fluitans CCAP 335.13	HQ654143 / HQ654209	HQ613246	Rindi et al. 2011
Klebsormidium fluitans CCAP 335.14	HQ654144 / HQ654210	HQ613247	Rindi et al. 2011
Klebsormidium fluitans SAG 9.96	AM490839 / AM490839	EU477438	Rindi et al. 2008, Sluiman et al. 2008
Klebsormidium mucosum SAG 8.96	EF372516 / EF372516	EU477445	Rindi et al. 2008, Sluiman et al. 2008
Klebsormidium nitens SAG 13.91	HQ654146 / HQ654212	HQ613248	Rindi et al. 2011
Klebsormidium nitens SAG 32.91	HQ654148 / HQ654214	EU477447	Rindi et al. 2011
Klebsormidium nitens SAG 52.91		EU477446	Rindi et al. 2008
Klebsormidium nitens SAG 335.2b	AM490844 / AM490844	AF408254	Karol et al. 2001; Sluiman et al. 2008
Klebsormidium scopulinum CCAP 335.15	HQ654152 / HQ654218	HQ613263	Rindi et al. 2011
Klebsormidium subtilissimum SAG 384.1	EF372517 / EF372517	EU477454	Rindi et al. 2008 ; Sluiman et al. 2008
Klebsormidium sp. GALW015300		EU477449	Rindi et al. 2008
Klebsormidium sp. GALW015463		EU477440	Rindi et al. 2008
Klebsormidium sp. GALW015468		EU477428	Rindi et al. 2008
Klebsormidium sp. GALW015499		EU477441	Rindi et al. 2008
Klebsormidium sp.K01	HE649310 / HE649310	HE649339	Škaloud & Rindi 2013
Klebsormidium sp.K02	HE649320 / HE649320	HE649349	Škaloud & Rindi 2013
Klebsormidium sp.K05	HE649315 / HE649315	HE649344	Škaloud & Rindi 2013
Klebsormidium sp.K06	HE649311 / HE649311	HE649340	Škaloud & Rindi 2013
Klebsormidium sp.K07	HE649316 / HE649316	HE649345	Škaloud & Rindi 2013
Klebsormidium sp.K09	HE649333 / HE649333	HE649362	Škaloud & Rindi 2013
Klebsormidium sp.K10	HE649336 / HE649336	HE649365	Škaloud & Rindi 2013
Klebsormidium sp.K11	HE649323 / HE649323	HE649352	Škaloud & Rindi 2013
Klebsormidium sp.K19	HE649329 / HE649329	HE649358	Škaloud & Rindi 2013
Klebsormidium sp.K22	HE649337 / HE649337	HE649366	Škaloud & Rindi 2013
Klebsormidium sp.K26	HE649324 / HE649324	HE649353	Škaloud & Rindi 2013
Klebsormidium sp.K28	HE649338 / HE649338	HE649367	Škaloud & Rindi 2013
Klebsormidium sp.K34	HE649314 / HE649314	HE649343	Škaloud & Rindi 2013
Klebsormidium sp.K37	HE649327 / HE649327	HE649356	Škaloud & Rindi 2013
Klebsormidium sp.K39	HE649331 / HE649331	HE649360	Škaloud & Rindi 2013
Klebsormidium sp.K40	HE649334 / HE649334	HE649363	Škaloud & Rindi 2013
Klebsormidium sp.K42	HE649322 / HE649322	HE649351	Škaloud & Rindi 2013
Klebsormidium sp.K43	HE649330 / HE649330	HE649359	Škaloud & Rindi 2013
Klebsormidium sp.K44	HE649332 / HE649332	HE649361	Škaloud & Rindi 2013
Klebsormidium sp. LUK 318	HQ654181 / HQ654247	HQ613251	Rindi et al. 2011
Klebsormdium sp. Namibia 5	HQ654162 / HQ654228	HQ613252	Rindi et al. 2011

Klebsormidium sp. SAG 2017	HQ654156 / HQ654222	HQ613254	Rindi et al. 2011
Klebsormidium sp. SAG 2108	HQ654157 / HQ654223	HQ613255	Rindi et al. 2011
Klebsormidium sp. SAG 2019	HQ654158 / HQ654224	HQ613256	Rindi et al. 2011
Klebsormidium sp. SAG 2065	EU434032 / EU434032	HQ613253	Mikhailyuk et al. 2008, Rindi et al. 2011
Klebsormidium sp.TR18	HQ654165 / HQ654231	HQ613257	Rindi et al. 2011
Klebsormidium sp. TR35	HQ654170 / HQ654236	HQ613260	Rindi et al. 2011
Klebsormidium sp. 14621.6	HQ654182 / HQ654248	HQ613250	Rindi et al. 2011
Klebsormidium sp. 14613.5e	HQ654176 / HQ654242	HQ613249	Rindi et al. 2011

3.2.Paper 2

Ryšánek D., Hrčková K. and Škaloud P. (2015)
Global ubiquity and local endemism of free-living terrestrial protists: phylogeographic assessment of the streptophyte alga *Klebsormidium*.
Environmental Microbiology 17: 689-698.

Global ubiquity and local endemism of free-living terrestrial protists: Phylogeographic assessment of the Streptophyte alga *Klebsormidium*

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ABSTRACT

Despite considerable research attention during the last 10 years, the distribution and biogeography of protists remain as highly controversial issues. The presumably huge population sizes and unlimited dispersal capabilities should result in protist ubiquity. However, recent molecular investigations suggest that protist communities exhibit strong biogeographic patterns. Here, we examined the biogeographic pattern of a very common green algal genus *Klebsormidium*. We evaluated the geographic distribution of *rbcL* genotypes for 190 isolates sampled in six sampling regions located in Europe, North America, and Asia. Measures of correlation between genetic and geographic distance matrices revealed a differential distribution pattern on two geographic levels. Globally, the populations were genetically homogeneous; locally, the genotypes were patchily distributed. We hypothesized that a local fine-scale structuring of genotypes may be caused by various ecological factors; in particular, by the habitat differentiation of particular genotypes. Our investigations also identified a large number of new, previously unrecognized lineages. A total of 44 genotypes were identified, and more than 66% of these were reported for the first time.

INTRODUCTION

Estimates of protist biogeography and diversity have become highly controversial topics during the last 10 years. It has been proposed that global protist species diversity is extremely high because most species have limited geographical distribution (Foissner, 1999, 2006). Conversely, there are arguments for ubiquitous distribution and unlimited dispersal of protists, which implies much lower total diversity compared to that of macroorganisms (Finlay *et al.*, 1996; Finlay 2002).

Supporters of the ubiquity theory argue that huge populations, small cell sizes, and almost unlimited dispersal of protists cause their cosmopolitan distribution (Fenchel, 1993; Finlay *et al.*, 1996, 2004). The ubiquity model suggests that microbial taxa of typical protist dimensions (approximately 20 μm in diameter) are capable of global dispersal (Wilkinson *et al.*, 2012). This dispersal is not limited by biogeographical barriers (Finlay, 2002), but it is driven by random events such as wind, ocean circulation, and transport on the bodies of migrating birds or animals (Fenchel and Finlay, 2004). Consequently, individual protist species occur wherever suitable environmental conditions are available.

The ubiquity model predicts a very low probability of local extinction in protist populations (Fenchel and Finlay, 2003). The consequence of very low local extinction coupled with extremely large population size is high local protist diversity and a low global diversity (Fenchel and Finlay, 2003, 2004). However, the reported low estimations of total protist species richness (Finlay and Fenchel, 1999) contradict the results of several molecular phylogenetic studies showing a large cryptic diversity in eukaryotic microorganisms (von der Heyden *et al.*, 2004; Šlapeta *et al.*, 2006; Simon *et al.*, 2008). Fenchel and Finlay (2006) postulated that the use of genetic data brought confusion into the estimations of real diversity in protists. They proposed that the variation in molecular markers reflects the accumulation of neutral mutations over historical time, rather than the existence of morphologically indiscernible, cryptic species.

Opponents of the ubiquity theory propose an extraordinarily high global diversity and endemicity of protist species. Foissner (2006) reviewed information that indicated restricted distributions for several protist groups and promoted the use of flagship species (i.e., species with conspicuous morphologies whose presence/absence could be easily demonstrated in samples) to demonstrate the endemism of several protists. For example, the morphologically distinct desmid species *Micrasterias hardyi* G. S. West and *Staurastrum victoriense* G. S. West have been reported only in Australia (Tyler, 1996). Accordingly, Foissner and colleagues proposed a moderate-endemicity model, which considers that some protist species

have cosmopolitan distributions, whereas other (perhaps rarer) protists have restricted distributions (Chao *et al.*, 2006; Foissner, 2006). This model has been corroborated by a number of subsequent studies (Van de Vijver et al., 2005; Bass *et al.*, 2007; Řezáčová and Neustupa, 2007; Robeson *et al.*, 2011; Bates *et al.*, 2012; Rengefords *et al.*, 2012;).

The vast majority of studies dealing with the biogeography of protists are based on aquatic organisms. However, terrestrial habitats could present more efficient migration barriers that lead to potentially high endemism in soil protists. Although Finlay *et al.* (2001) reported a cosmopolitan distribution for the majority of soil protists, several studies revealed significant biogeographical patterns in the distribution of terrestrial protist communities (Boenigk *et al.*, 2005; Bass *et al.*, 2007; Bates *et al.*, 2012; Ragon *et al.*, 2012). A recently published high-throughput pyrosequencing investigation of terrestrial microbial communities revealed that the community structure could be significantly influenced by environmental factors (Bates *et al.*, 2012). Indeed, the terrestrial communities were strongly structured by climatic conditions that affect the annual soil moisture availability.

Investigating the community structure provides great insight into the spatial patterns of protist variation. However, focusing on narrow evolutionary lineages could provide a robust framework for evaluating biogeographical patterns at high phylogenetic resolution. In the present study, we investigated the global biogeography of the terrestrial cosmopolitan genus *Klebsormidium*. This genus occurs in a wide range of habitats and represents one of the most abundant autotrophic organisms found in various aeroterrestrial microbial communities (Deason, 1969; Handa *et al.*, 1991; Nakano *et al.*, 1991; Baldwin and Whitton, 1992; Rindi and Guiry, 2003; 2004; Smith *et al.*, 2004; Barberousse *et al.*, 2006; Rindi *et al.*, 2008). *Klebsormidium* has widespread cosmopolitan distribution from polar to tropical regions (Ramanathan, 1964; Lee and Wee, 1982; Broady, 1996; Lokhorst, 1996; John, 2002; 2003).
Several molecular genetics studies demonstrated that diversity within the genus *Klebsormidium* is far greater than that expected on the basis of morphological features, and concluded that the traditional phenotypic species concept is insufficient (Rindi *et al.*, 2008, 2011). For example, one of the most commonly identified species, *K. flaccidum*, is highly polyphyletic and occurring in five different clades. These cryptic lineages have been recently recognized to be ecologically differentiated (Škaloud and Rindi, 2013).

The general aim of this study was to examine the continental-scale biogeographical pattern of a single evolutionary lineage of terrestrial protists, the green algal genus *Klebsormidium*. We used *rbcL* sequences for molecular screening of the isolated strains because they have better resolution than the rapidly evolving ITS rDNA (Rindi *et al.* 2011).

We investigated whether the global dispersal of particular *rbcL* genotypes occurs faster than their divergence. We also assessed whether the overall genetic diversity of a molecularly well characterized protist lineage increases by a comprehensive world-wide sampling.

Results

The samples analyzed in this work were collected in six regions located in Europe, North America, and Asia. Within each region, terrestrial, epilithic, and corticolous samples were collected at 2-4 sampling sites. A total of 190 *Klebsormidium* colonies were isolated from the samples, and the *rbcL* gene of each colony was sequenced (Supplementary Table 1). The majority of sequences (87) were obtained from samples collected in Europe (Wales, 63; Czech Republic, 24), 65 sequences were obtained from samples collected in the USA (Washington, 21; Ohio, 22; Connecticut, 22), and the remaining 38 sequences were obtained from samples originating in Japan.

The sequences yielded a total of 44 unique *rbcL* genotypes, indicating very high genotype diversity in the dataset. Of the 44 unique genotypes, 23 were represented by a single sequence. The Bayesian phylogenetic analysis (Fig. 1) revealed a significant genetic divergence among the genotypes. They were recovered in almost all superclades sensu Rindi *et al.* (2011), with the exception of the superclades A and G. These represent a morphologically distinct genus *Interfilum* and an ecologically defined lineage of desert soil-crust inhabitants. Although several novel genotypes were recognized, none of them formed a new superclade. The majority of genotypes were inferred within superclade E, a heterogeneous assemblage of a number of morphologically and ecologically different lineages (Škaloud and Rindi, 2013). With the exception of the clade E6 (*K. subtillisimum*), the genotypes were inferred in all clades recognized within the superclade E by Rindi *et al.* (2011).

To achieve the best insight into the biogeography of particular *Klebsormidium* lineages, we constructed an additional phylogenetic tree based on only those sequences generated in this study (Fig. 2). In general, all inferred superclades were detected in at least two biogeographic regions. Within the superclade B+C, the sequences originated primarily from the USA (76%), whereas the European and Japanese sequences were less abundant (14% and 10%, respectively). Superclade D contained strains isolated almost exclusively from Europe (96%), with a single, distantly related sequence that originated from Japan. Superclade E was present in all six investigated regions. It contained the majority of all obtained sequences (76%) and genotypes (73%). In general, superclade E did not predominant

in any of the three continents; it represented 33%, 43%, and 24% of North American, European, and Asian sequences, respectively. However, when focusing on individual nested clades, some biogeographical patterns were evident. For example, clade E1 was composed of two Japanese genotypes (E1A and E1B), but also contains genotypes from New Zealand and Great Britain mainly from freshwater habitats. Similarly, the clade E5 comprised solely of European sequences (E5A) found in both investigated areas, the Czech Republic and Wales, and previously one sequence was found in Germany. In this clade is one sequence from Florida, USA, but different genotype. Finally, in our study the clade E4 was found in USA and Europe only, though a relatively high number of sequences ware obtained (11 from Europe, 8 from USA), but previously one sequences was found in Australia. The remaining two clades, (E2 and E3) had cosmopolitan distributions. Superclade F was formed by only two strains isolated from Europe and USA.

The regional distribution of the genotypes within each inferred clade is shown in Fig. 3. The highest genotypic diversity was found within clade E2, which also contained the most frequently occurring genotype E2C. This cosmopolitan genotype, represented by 22 genotypes, occurred in all but one of the investigated regions. Almost all genotypes represented by more than eight *rbcL* sequences were shared between at least two continents, with the two exceptions, first of an endemic European genotype DB that was represented by 18 sequences, second of an endemic American genotype E2F with 8 sequences (7 from Cleveland, and 1 from Barlow). In general, approximately half of 21 genotypes represented by at least two sequences had a cosmopolitan distribution. The genotypes were less shared among particular areas within a single continent than among continents. The data show that nine American genotypes were found in Europe and/or Asia, whereas only two of these genotypes were present at two different American regions. Indeed, no genotype was shared among the three American regions. Similarly, nine European genotypes were found in other continents, whereas only four genotypes occurred in both European regions.

To examine the effect of geographic distance on genetic structure and the relative contribution of gene flow to geographic structuring, isolation by distance (IBD) analyses were performed on the following two datasets: (1) the six sampling regions, comprising the Czech Republic, Wales, Washington, Ohio, Connecticut, and Japan; and (2) the 16 sampling sites. No significant correlation was detected among the six sampling regions (p = 0.7261). However, a positive, significant correlation between the genetic and geographic distances was detected among the 16 sampling sites (Fig. 4). The significance of the observed correlation

was verified using the Mantel test (Z = 102.4818, r = 0.1188, p = 0.0382). Reduced major axis (RMA) regression analysis yielded a value of R^2 = 0.0141 (slope = 0.1123).

DISCUSSION

Distribution of *Klebsormidium* lineages

Although the global biogeographic distributions of several aquatic protists have been documented (Finlay and Clarke, 1999; Sabbe *et al.*, 2001; Montresor *et al.*, 2003; Šlapeta *et al.*, 2006; Neustupa and Řezáčová, 2007), the biogeography of aeroterrestrial organisms remained largely unclear. In this study, we analyzed a relatively large number of aeroterrestrial algae belonging to the genus *Klebsormidium* to assess its distribution pattern in the Northern temperate zone. In total, we collected 190 strains originating from three different continents. The results demonstrated that a majority of genotypes represented by several isolates have a cosmopolitan distribution, whereas only a few genotypes were isolated in a single continent.

Our data indicate that the genus *Klebsormidium* has a generally cosmopolitan distribution with long-distance gene flow of the aeroterrestrial isolates. The small cell size and great abundance could result in unlimited dispersal that is unrestricted by geographical boundaries. Although filamentous, *Klebsormidium* species easily disintegrate into fragments containing a few cells (Škaloud, 2006). These fragments can spread easily due to random events, such as hurricanes or wind currents. Indeed, viable *Klebsormidium* cells have been detected in lower troposphere air samples (van Overeem, 1937). Dispersal by win (airborne) is usual way of distribution mainly for aeroterrestrial algae (Sharma *et al.,* 2007). Crucial factor for airborne is survive desiccation and UV radiation. Physiological studies terrestrial algae provide mechanisms of protection and adaptation to high UV radiation (Lud *et al.,* 2001; Hughes, 2006; Holzinger and Lütz, 2006; Karsten *et al.,* 2007; Pichrtová *et al.,* 2013), and desiccation (Haübner et al., 2006; Lüttge and Büdel, 2010; Karsten and Holzinger, 2012, 2014). On the other side, freshwater algae are usually transport by birds (Schlichting, 1960; Atkinson, 1970; Figuerola and Green, 2002), and also by mammals (Maguir, 1963; Roscher, 1967). Dispersal out of the water, from one water body to another normally involves changes of the environment from water to air and back to water again, with danger of desiccation. If the transport takes place in the intestine of an animal, there is no danger of desiccation, but cells are exposed to digestion juices (Kristiansen, 1996, 2008). For those types of transport for alga is necessary produce spores or cysts which are more able survive desiccation nor to survive transport through the digestive systems of wildfowl (Atkinson, 1971; Foissner, 2006,

2008). For those reasons there are some studies provided an evidence of limited dispersal capacities for freshwater alga (Vyverman *et al.,* 2007; *Evans et al.,* 2009; Souffreau *et al.,* 2010; Rangefors *et al.,* 2012).

In a recently published phylogenetic investigation of this genus, Rindi *et al.* (2011) discussed a limited distribution of several identified clades, based on their investigation of 87 sequenced strains originating from five continents. However, our extended sampling revealed a cosmopolitan distribution of all of these presumably endemic lineages. For example, the proposed Eastern European superclade B has now been identified in North America. Similarly, our sampling revealed the apparent cosmopolitan distribution of superclade C, which was proposed to be restricted to Western Europe. In fact, it is very likely that the presumably limited distribution of some *Klebsormidium* genotypes in our study may be due to their limited sampling. Similar trends were reported by Kristiansen (2008), who stressed that almost all *Mallomonas* taxa originally started as endemics but sooner or later lost this status due to more intensive research.

In our study, only 4 of the 16 most frequent genotypes were recognized to have limited geographical distribution. The two closely-related genotypes E2J and E2K were restricted to the East Asian region, and the remaining two genotypes were identified only in North America (E2F) and Europe (DB). The distribution of those four lineages strictly contradicts a fundamental pattern of random spatial dispersal of ubiquitous organisms, characterized by correlated local and global species richness (Finlay and Clarke, 1999; Finlay *et al.*, 2001). Actually, all genotypes were very numerous on a local scale, but very rare on a global scale. However, these atypical distribution patterns could be explained by strict ecological preferences for environmental conditions or habitat types. For example, the genotype DB was represented by 18 isolates sampled in a very restricted area of southern Wales (Supplementary Table S1). Both sampling sites of this genotype were located on the southern edge of the Brecon Beacons National Park (Wales, UK), on the rocks of the Coal Measures (Barclay *et al.*, 1989). Therefore, an ecologically restricted habitat preference of the DB genotype is the most plausible explanation of the observed distribution pattern. Recently, strong ecological differentiation of particular *Klebsormidium* lineages has been reported by Škaloud and Rindi (2013). Ecological differentiation of closely-related lineages has been reported for many different protist taxa (Rindi and Guiry, 2002; Rindi *et al.*, 2007; Peksa and Škaloud, 2011; Moniz *et al.*, 2012).

The IBD analysis did not prove any significant correlation between the genetic and geographic distance among the six investigated regions. However, the IBD analysis of genetic

sequence distributions among all 16 sampled sites revealed a weakly significant relationship between the genetic and geographic distances (Fig. 4). Dissimilar results of those two analyses imply a small-scale IBD patterns within each of the investigated regions. This indicates that the populations were homogeneous on a global scale, whereas the genotypes were patchily distributed on a local scale. Although this pattern might be viewed as an artifact of fragmentation of localities into smaller units, a fine-scale structuring of genotypes may be caused by various ecological factors, in particular by the habitat differentiation of particular genotypes (Škaloud and Rindi, 2013).

Genetic diversity

The data presented in this study expand our knowledge of the overall diversity and commonness of particular lineages within the genus *Klebsormidium.* Molecular investigations of nominal, morphologically defined protist taxa usually detect the presence of substantial genetic diversity and the presence of a large number of hidden species (von der Heyden *et al.*, 2004; Šlapeta *et al.*, 2006; Simon *et al.*, 2008). The genetic diversity within the genus *Klebsormidium* has been investigated in detail by Rindi *et al.* (2011) and Škaloud and Rindi (2013), who identified seven main superclades and 24 well-supported clades. Although we did not discover any novel superclade, our investigations led to the identification of a large number of new, previously unrecognized genotypes. From a total number of 44 identified genotypes, more than 66% were reported for the first time. Although the majority of published *Klebsormidium* sequences originated from various European isolates, we identified several novel lineages in Wales, including one within the most commonly sampled genotype DB.

We identified obvious differences in the frequency of genotype occurrences of particular *Klebsormidium* superclades. Consistent with the study of Rindi *et al.* (2011), we found that lineage E represents the most commonly sampled superclade. In particular, 76% of all investigated strains belonged within clade E2. Although we analyzed approximately 200 *Klebsormidium* strains from three different continents, we did not sample a single genotype belonging to superclade G sensu Rindi *et al.* (2011). The superclade G could exhibit ecological preferences for specific environmental conditions or habitat types because it was originally isolated from biotic crusts of arid soils in South Africa (Rindi *et al.*, 2011). This superclade seems to be ecologically restricted to arid soil environments, in particular to the soil crusts of warm and arid desert and subdesert areas (Rindi *et al.*, 2011).

EXPERIMENTAL PROCEDURES

Sample collection and culturing

The samples were collected in six regions located in mixed forests of the Northern temperate zone on three continents: two in Europe (Wales and Czech Republic), three in North America (Washington, Ohio, and Connecticut), and one in Asia (Japan). For each region, 2–4 different sampling sites were selected for collecting samples. At each sampling site, a number of samples were collected from a broad range of terrestrial, epilithic, and corticolous substrates. Samples were mixed together and transferred in sterile plastic bags into the laboratory. Samples were inoculated on two agar plates with modified Bold's Basal Medium (Bischoff and Bold, 1963) at 20°C under a 14 h/10 h light/dark regime using photon irradiance of approximately 30–50 μmol photons m^{-2} s^{-1} provided by 18 W cool fluorescent tubes (Philips TLD 18W/33). After 5–10 weeks, the agar plates were checked for the presence of *Klebsormidium* microcolonies.

DNA isolation

For DNA isolation, identified *Klebsormidium* microcolonies were transferred to polymerase chain reaction (PCR) tubes containing 100 μl of InstaGene matrix (Bio-Rad Laboratories, Hercules, CA, USA). The cells were then mechanically disrupted by shaking for 6 min in the presence of glass beads (3 mm diameter; Sigma-Aldrich) in Mixer Mill MM 400 (Retsch, Haan, Germany). Subsequently, the samples were incubated at 56°C for 30 min (under continuous mixing at 700 rpm), and then heated to 99°C for 8 min. After vortexing, the tubes were centrifuged at 12,000 rpm for 2 min, and the supernatant was directly used as a PCR template.

PCR and sequencing

Sequences of the *rbcL* gene were obtained by PCR amplification using a Touchgene Gradient cycler (Techne). The *rbcL* gene was amplified using the newly designed primer KF590 (5′-GAT GAA AAC GTA AAC TCT CAG C-3′) and the primer *rbcL*-KR2 (5′-GGT TGC CTT CGC GAG CTA-3′; Škaloud and Rindi, 2013). Each 20 μL PCR reaction contained 13.9 μL of sterile Milli-Q water, 2 μL of PCR buffer (Sigma), 2 μL of $MgCl_2$, 0.4 μL of dNTP (10 μM), 0.25 μL of primers (25 pmol ml^{-1}), 0.2 μL of AmpliTaq Gold DNA Polymerase (Applied Biosystems), and 1 μl of DNA. The PCR amplification, purification, and sequencing were performed as described in Škaloud and Rindi (2013). Sequencing reads were assembled and edited by use of SeqAssem software (Hepperle, 2004).

Sequence analyses

For phylogenetic analyses, the newly obtained *Klebsormidium rbcL* sequences and the sequences available in NCBI GenBank database were used to generate the alignment. The final alignment of 606 base pairs (bp) was constructed by ClustalW (Thompson *et al.*, 1994) using MEGA 5.05 (Tamura *et al.*, 2011). The aligned data set was analyzed by maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses as described previously (Škaloud and Rindi, 2013), using the GTR + I + G evolutionary model selected according to the Akaike Information Criterion computed in PAUP/Mr.Modeltest 2.3 (Nylander, 2004). Genotype networks were obtained by statistical parsimony analysis in TCS v1.21 (Clement *et al.*, 2000), using the 95% plausible connection limit. Isolation by distance analyses were performed using the IBDWS 3.23 program (Jensen *et al.*, 2005). Pairwise matrices of geographical distance (log geographical distance) and PhiST (incorporates sequence distance information) were compared using a Mantel test for matrix correlation (Mantel, 1967), with significance assessed by 10,000 randomizations of the genetic distance matrix.

ACKNOWLEDGMENTS

The authors would like to thank Yvonne Němcová and Magda Škaloudová for their kind support during sampling. This research was supported by a grant from the Czech Science Foundation (no. P506/12/0955). Sampling in USA was supported by the Fulbright-Masaryk Scholarship.

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FIGURES

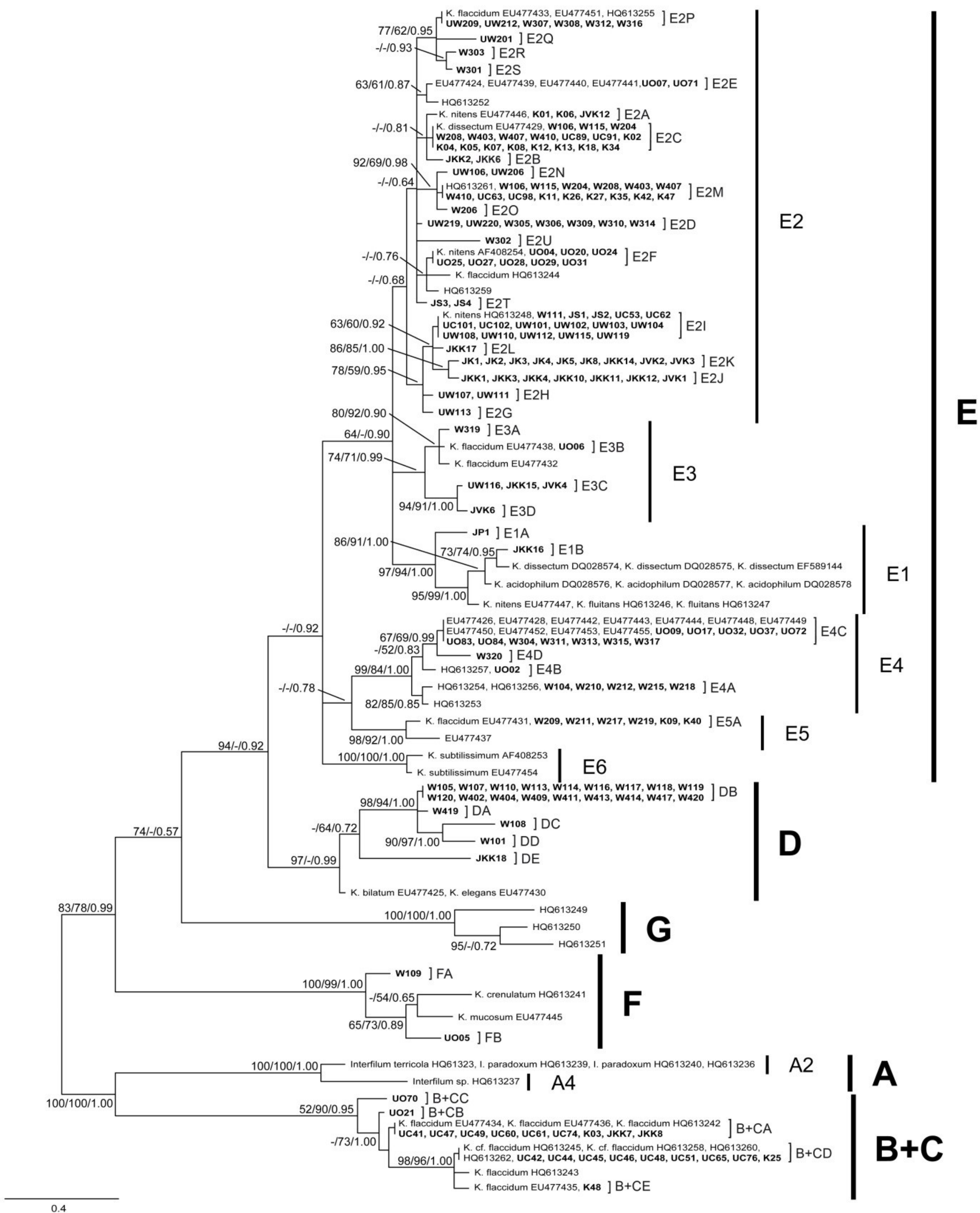

Figure 1. Phylogenetic relationships among *Klebsormidium* lineages. The phylogenetic unrooted tree was inferred based on a Bayesian analysis of *rbcL* sequences. Values at the nodes indicate statistical support estimated by three methods: maximum parsimony bootstrap (left), maximum likelihood bootstrap (middle), and MrBayes posterior node probability (right). Sequences determined in this study are given in bold. Scale bar represents an expected number of substitutions per site.

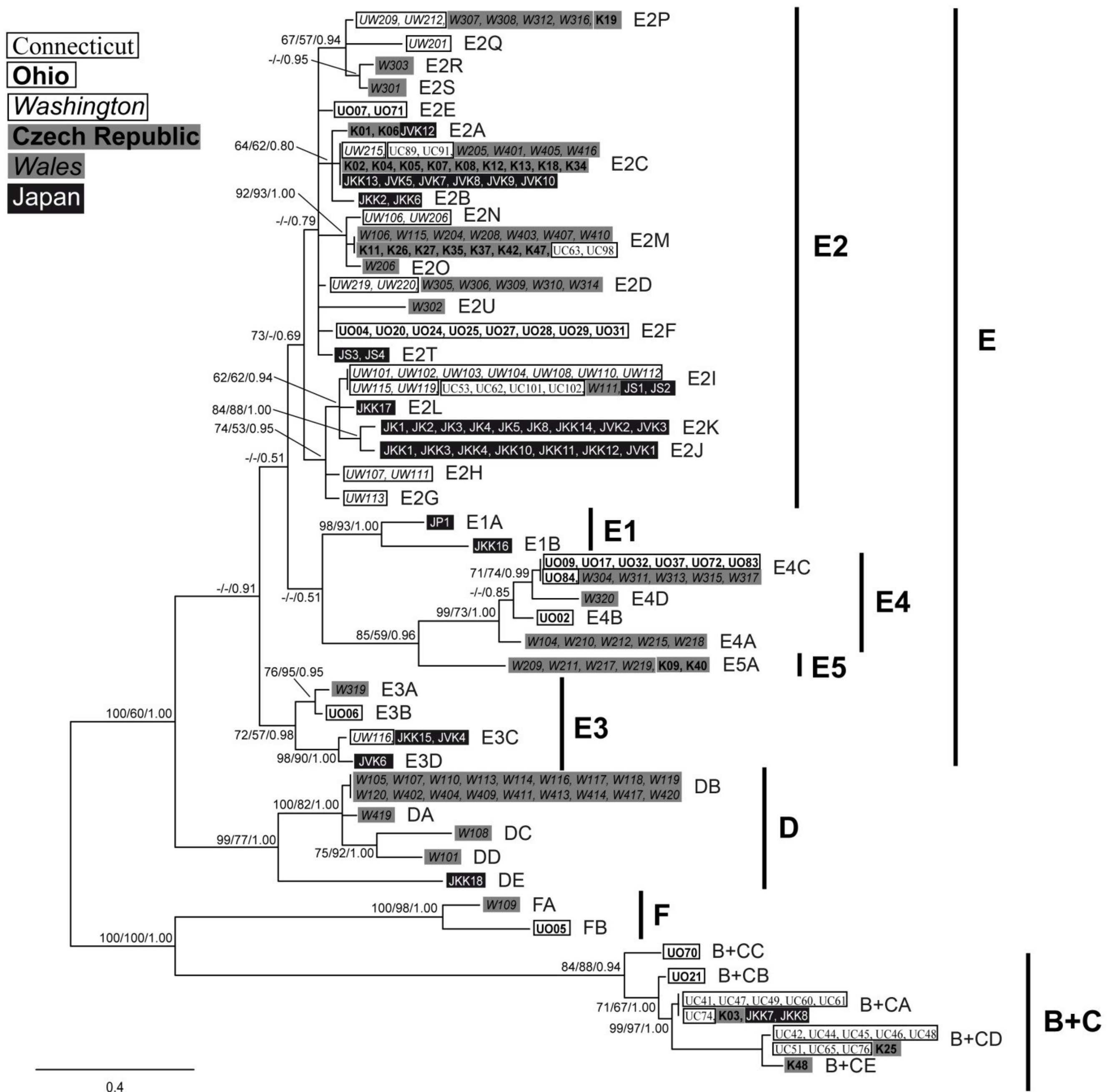

Figure 2. Phylogenetic relationships among *Klebsormidium* genotypes obtained in this study. The phylogenetic unrooted tree was inferred based on a Bayesian analysis of *rbcL* sequences. Values at the nodes indicate statistical support estimated by three methods: maximum parsimony bootstrap (left), maximum likelihood bootstrap (middle), and MrBayes posterior node probability (right). Geographic origin of particular strains is indicated. Scale bar represents an expected number of substitutions per site.

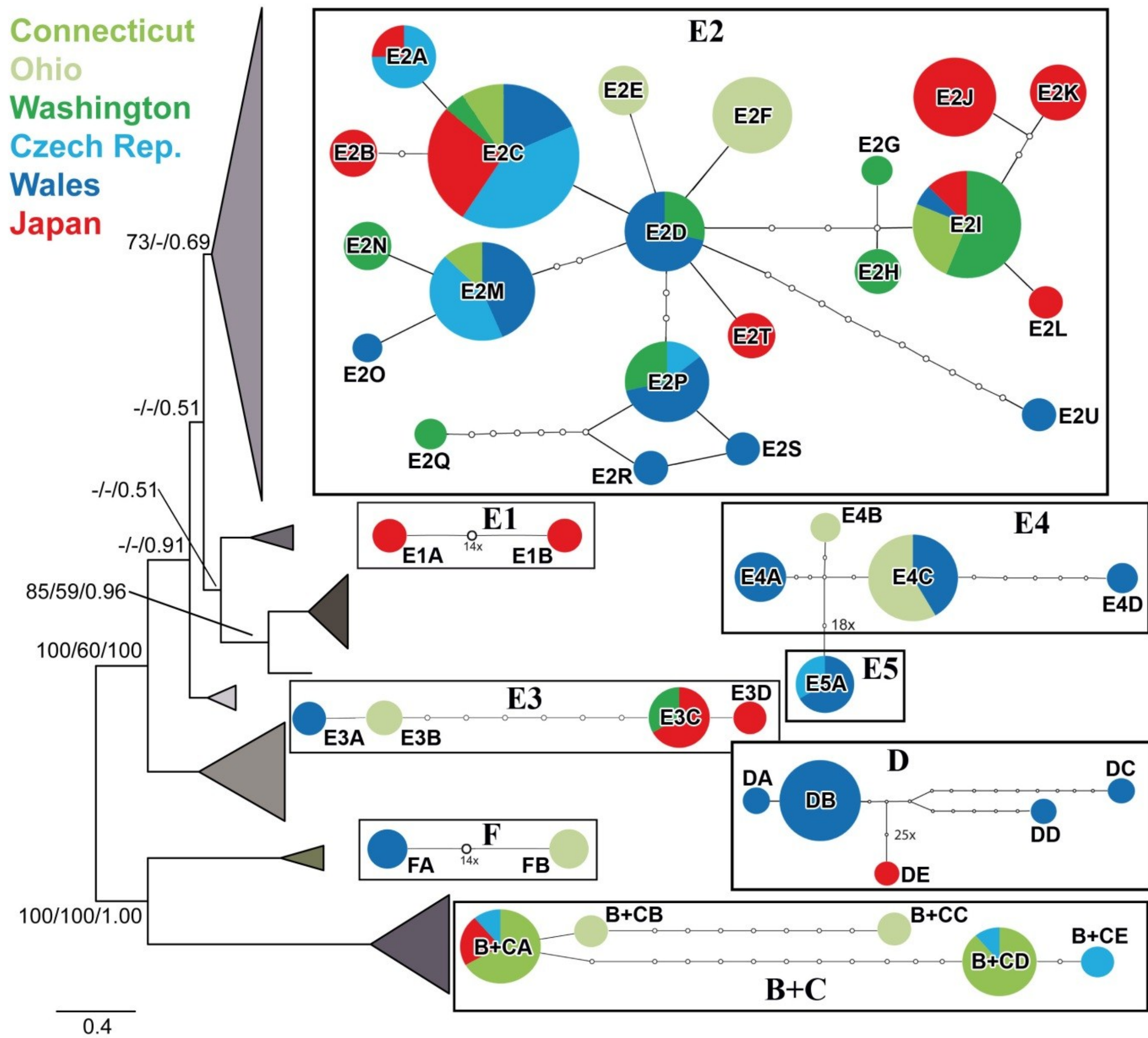

Figure 3. Statistical parsimony haplotype network representing genealogical relationships among 44 *Klebsormidium* genotypes of the *rbcL* gene. Genotypes are colored according to the respective sampling region (American and European regions are given in green and blue shades, respectively). The sizes of circles representing genotypes reflect the number of sequences that share a genotype. Inferred intermediate haplotypes that were either not sampled or are extinct are represented by small non-colored circles. Genotypes belonging to eight major inferred clades are outlined by the black frames. Each of the genotypes is coded by a unique identifier reflecting its clade affiliation.

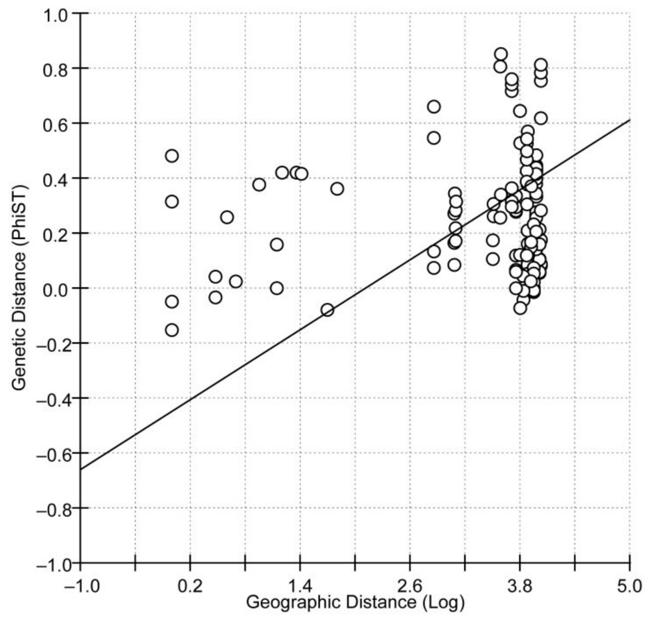

Figure 4. Scatter plot of pairwise genetic distance (PhiST) versus geographic distance (log geographic distance in km) of 16 sampling sites. A significant correlation between the distances was detected using the Mantel test ($Z = 102.4818$, $r = 0.1188$, $p = 0.0382$).

SUPPLEMENTARY DATA

Table 1. List of the *Klebsormidium* strains used in this study, including collection data, accession numbers of *rbc*L sequences.

Herbarium/culture	Clade affiliation	Locality	Habitat	Accession No. rbcL	Species assignation
SAG 2101	A	Ukraine	in cracks of granite outcrop	HQ613236	sp.
SAG 2100	A	Haute Ardenne, Belgium	soil from oak tree forest	HQ613238	terricola
SAG 4.85	A	Bressanone, Italy	soil	HQ613239	paradoxum
SAG 338.1	A	Epping, near London, U.K.	soil from beech forest	HQ613240	paradoxum
SAG 2147	A	Czech Republic	soil	HQ613237	sp.
UO70	B + C	Cleveland, Ohio, USA	rock	x	sp.
UO21	B + C	Cleveland, Ohio, USA	concreate (wall)	x	sp.
SAG 335.7	B + C	Smaaland, Sweden	freshwater	EU477434	flaccidum
SAG 12.91	B + C	Sojovice reservoir, Czech Republic	freshwater	EU477436	flaccidum
SAG 2307	B + C	Germany	clayey soil on field with beets	HQ613242	flaccidum
UC41	B + C	Connecticut, USA	concreate (wall)	x	sp.
UC47	B + C	Connecticut, USA	concreate (wall)	x	sp.
UC49	B + C	Connecticut, USA	concreate (wall)	x	sp.
UC60	B + C	Connecticut, USA	soil	x	sp.
UC61	B + C	Connecticut, USA	soil	x	sp.
UC74	B + C	Connecticut, USA	rock	x	sp.
K03	B + C	České středohoří Mts., Czech Republic	rock (pholonite)	x	sp.
JKK7	B + C	Nikko, Japan	rock	x	sp.
JKK8	B + C	Nikko, Japan	rock	x	sp.
ACKU 800	B + C	Boguslav, Ukraine	granite outcrop	HQ613245	flaccidum
ACKU 379	B + C	Ukraine	granite outcrop	HQ613258	flaccidum
TR 35	B + C	Zhytomir, Ukraine	granite outcrop	HQ613260	sp.

ACKU 801	B + C	Boguslav, Ukraine	granite outcrop	HQ613262	sp.
UC42	B + C	Connecticut, USA	concreate (wall)	x	sp.
UC44	B + C	Connecticut, USA	concreate (wall)	x	sp.
UC45	B + C	Connecticut, USA	concreate (wall)	x	sp.
UC46	B + C	Connecticut, USA	concreate (wall)	x	sp.
UC48	B + C	Connecticut, USA	concreate (wall)	x	sp.
UC51	B + C	Connecticut, USA	concreate (wall)	x	sp.
UC65	B + C	Connecticut, USA	soil	x	sp.
UC76	B + C	Connecticut, USA	bark	x	sp.
K25	B + C	České středohoří Mts., Czech Republic	rock (pholonite)	x	sp.
Lira7	B + C	Russia	freshwater	HQ613243	flaccidum
SAG 7.91	B + C	SSSR	freshwater	EU477435	flaccidum
K48	B + C	Drahamské údolí, Czech Republic	rock	x	sp.
W105	D	Glynneath, Wales	rock	x	sp.
W107	D	Glynneath, Wales	rock	x	sp.
W110	D	Glynneath, Wales	rock	x	sp.
W113	D	Glynneath, Wales	rock	x	sp.
W114	D	Glynneath, Wales	rock	x	sp.
W116	D	Glynneath, Wales	rock	x	sp.
W117	D	Glynneath, Wales	rock	x	sp.
W118	D	Glynneath, Wales	rock	x	sp.
W119	D	Glynneath, Wales	rock	x	sp.
W120	D	Glynneath, Wales	rock	x	sp.
W402	D	Merthyr Tydfil, Wales	rock	x	sp.
W404	D	Merthyr Tydfil, Wales	rock	x	sp.
W409	D	Merthyr Tydfil, Wales	rock	x	sp.
W411	D	Merthyr Tydfil, Wales	rock	x	sp.

W413	D	Merthyr Tydfil, Wales	rock	x	sp.
W414	D	Merthyr Tydfil, Wales	rock	x	sp.
W417	D	Merthyr Tydfil, Wales	rock	x	sp.
W419	D	Merthyr Tydfil, Wales	rock	x	sp.
W420	D	Merthyr Tydfil, Wales	rock	x	sp.
W101	D	Glynneath, Wales	rock	x	sp.
W108	D	Glynneath, Wales	rock	x	sp.
JKK18	D	Nikko, Japan	rock	x	sp.
SAG 5.96	D	Poppel, Belgium	bank at a brooklet	EU477425	bilatum
SAG 7.96	D	Staverden, Netherlands	bark of oak tree	EU477430	elegans
JP1	E1	Nikko, Japan	soil	x	sp.
JKK16	E1	Nikko, Japan	rock	x	sp.
Novis K25	E1	river Acheron, New zealand	freshwater	DQ028574	dissectum
Novis K37	E1	river Ryton, New Zealand	freshwater	DQ028575	dissectum
Novis LCR-K2	E1	Philistinem Mts., New Zealand	soil	EF589144	dissectum
Novis K48	E1	stream Agility, New Zealand	freshwater	DQ028576	acidophilum
Novis KM	E1	Millerton, New Zealand	freshwater (acidic)	DQ028577	acidophilum
Novis KRIV	E1	Sullivan, New Zealand	freshwater (acidic)	DQ028578	acidophilum
SAG 32.91	E1	river Gannel, Great Britain	freshwater	EU477447	nitens
CCAP 335.13	E1	river Hayle, Great Britain	freshwater	HQ613246	fluitans
CCAP 335.14	E1	river Hayle, Great Britain	freshwater	HQ613247	fluitans
SAG 38.91	E2	Witzenhausen, Germany	x	EU477433	flaccidum
GALW015451	E2	Porto, Portugal	base of urban wall	EU477451	sp.
SAG 2108	E2	x	x	HQ613255	sp.
GALW015473	E2	Bordeaux, France	base of urban wall	EU477427	sp.
UW209	E2	Seattle, Washington, USA	rock	x	sp.
UW212	E2	Seattle, Washington, USA	rock	x	sp.

W307	E2	Talybont, Wales	rock	x	sp.
W308	E2	Talybont, Wales	rock	x	sp.
W312	E2	Talybont, Wales	rock	x	sp.
W316	E2	Talybont, Wales	rock	x	sp.
K19	E2	Koleč, Czech Republic	concreate (moss vegetation on wall)	x	sp.
UW201	E2	Seattle, Washington, USA	rock	x	sp.
W301	E2	Talybont, Wales	rock	x	sp.
W303	E2	Talybont, Wales	rock	x	sp.
GALW015340	E2	Bergen, Norway	base of urban wall	EU477424	sp.
GALW015548	E2	Greskop, South Africa	base of wall in shaded corner	EU477439	sp.
GALW015463	E2	Hamburg, Germany	concreate (wall)	EU477440	sp.
GALW015499	E2	Konstans, Germany	concreate (wall)	EU477441	sp.
UO07	E2	Cleveland, Ohio, USA	rock	x	sp.
UO71	E2	Cleveland, Ohio, USA	rock	x	sp.
Namibia 5	E2	Namibia	artificial stony substratum	HQ613252	sp.
SAG 52.91	E2	Mors island, Denmark	soil	EU477446	nitens
K01	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649339	sp.
K06	E2	České středohoří Mts., Czech Republic	moss	HE649340	sp.
K20	E2	Milská stráň, Czech Republic	soil	HE649342	sp.
JVK12	E2	Nikko, Japan	rock	x	sp.
SAG 2155	E2	Col du Bussang, France	on moist sandy soil of forest track	EU477429	dissectum
UW215	E2	Seattle, Washington, USA	rock	x	sp.
JVK5	E2	Nikko, Japan	rock	x	sp.
JVK7	E2	Nikko, Japan	rock	x	sp.
JVK8	E2	Nikko, Japan	rock	x	sp.
JVK9	E2	Nikko, Japan	rock	x	sp.
JVK10	E2	Nikko, Japan	rock	x	sp.
JKK13	E2	Nikko, Japan	rock	x	sp.

W205	E2	Brecon, Wales	rock	x	sp.
W401	E2	Merthyr Tydfil, Wales	rock	x	sp.
W405	E2	Merthyr Tydfil, Wales	rock	x	sp.
W416	E2	Merthyr Tydfil, Wales	rock	x	sp.
UC89	E2	Connecticut, USA	bark	x	sp.
UC91	E2	Connecticut, USA	bark	x	sp.
K02	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649349	sp.
K05	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649344	sp.
K07	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649345	sp.
K08	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649346	sp.
K12	E2	České středohoří Mts., Czech Republic	soil	HE649350	sp.
K13	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649347	sp.
K18	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649348	sp.
K34	E2	Velké Přílepy, Czech Republic	rock (lydite)	HE649343	sp.
JKK2	E2	Nikko, Japan	rock	x	sp.
JKK6	E2	Nikko, Japan	rock	x	sp.
UW206	E2	Seattle, Washington, USA	rock	x	sp.
UW106	E2	Seattle, Washington, USA	rock	x	sp.
ACKU 799	E2	Boguslav, Ukraine	granite outcrop	HQ613261	sp.
W106	E2	Glynneath, Wales	rock	x	sp.
W115	E2	Glynneath, Wales	rock	x	sp.
W204	E2	Brecon, Wales	rock	x	sp.
W208	E2	Brecon, Wales	rock	x	sp.
W403	E2	Merthyr Tydfil, Wales	rock	x	sp.
W407	E2	Merthyr Tydfil, Wales	rock	x	sp.
W410	E2	Merthyr Tydfil, Wales	rock	x	sp.
K11	E2	České středohoří Mts., Czech Republic	soil	HE649352	sp.

K26	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649353	sp.
K27	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649354	sp.
K35	E2	Drahamské údolí, Czech Republic	rock (schist)	HE649355	sp.
K37	E2	Drahamské údolí, Czech Republic	rock (schist)	HE649356	sp.
K42	E2	České středohoří Mts., Czech Republic	rock (pholonite)	HE649351	sp.
K47	E2	Drahamské údolí, Czech Republic	rock (schist)	HE649357	sp.
UC63	E2	Connecticut, USA	soil	x	sp.
UC98	E2	Connecticut, USA	concreate	x	sp.
W206	E2	Brecon, Wales	rock	x	sp.
UW219	E2	Seattle, Washington, USA	rock	x	sp.
UW220	E2	Seattle, Washington, USA	rock	x	sp.
W305	E2	Talybont, Wales	rock	x	sp.
W306	E2	Talybont, Wales	rock	x	sp.
W309	E2	Talybont, Wales	rock	x	sp.
W310	E2	Talybont, Wales	rock	x	sp.
W314	E2	Talybont, Wales	rock	x	sp.
W302	E2	Talybont, Wales	rock	x	sp.
SAG 335.2b	E2	Barlow, USA	freshwater	AF408254	nitens
UO04	E2	Cleveland, Ohio, USA	rock	x	sp.
UO20	E2	Cleveland, Ohio, USA	concreate	x	sp.
UO24	E2	Cleveland, Ohio, USA	concreate	x	sp.
UO25	E2	Cleveland, Ohio, USA	concreate	x	sp.
UO27	E2	Cleveland, Ohio, USA	concreate	x	sp.
UO28	E2	Cleveland, Ohio, USA	concreate	x	sp.
UO29	E2	Cleveland, Ohio, USA	concreate	x	sp.
UO31	E2	Cleveland, Ohio, USA	concreate	x	sp.
SAG 37.91	E2	Lake Titicaca, Peru	freshwater	HQ613244	flaccidum
TR 31	E2	Australia	soil	HQ613259	sp.

JS3	E2	Nikko, Japan	road	x	sp.
JS4	E2	Nikko, Japan	road	x	sp.
SAG 13.91	E2	Tehoa, New Zealand	soil	HQ613248	nitens
UW101	E2	Seattle, Washington, USA	rock	x	sp.
UW102	E2	Seattle, Washington, USA	rock	x	sp.
UW103	E2	Seattle, Washington, USA	rock	x	sp.
UW104	E2	Seattle, Washington, USA	rock	x	sp.
UW108	E2	Seattle, Washington, USA	rock	x	sp.
UW110	E2	Seattle, Washington, USA	rock	x	sp.
UW112	E2	Seattle, Washington, USA	rock	x	sp.
UW115	E2	Seattle, Washington, USA	rock	x	sp.
UW119	E2	Seattle, Washington, USA	rock	x	sp.
W111	E2	Glynneath, Wales	rock	x	sp.
UC53	E2	Connecticut, USA	soil	x	sp.
UC62	E2	Connecticut, USA	soil	x	sp.
UC101	E2	Connecticut, USA	soil	x	sp.
UC102	E2	Connecticut, USA	soil	x	sp.
JS1	E2	Nikko, Japan	road	x	sp.
JS2	E2	Nikko, Japan	road	x	sp.
JKK17	E2	Nikko, Japan	rock	x	sp.
JK1	E2	Nikko, Japan	rock	x	sp.
JK2	E2	Nikko, Japan	rock	x	sp.
JKK14	E2	Nikko, Japan	rock	x	sp.
JVK2	E2	Nikko, Japan	rock	x	sp.
JVK3	E2	Nikko, Japan	rock	x	sp.
JKK1	E2	Nikko, Japan	rock	x	sp.
JKK3	E2	Nikko, Japan	rock	x	sp.

JKK4	E2	Nikko, Japan	rock	x	sp.
JKK10	E2	Nikko, Japan	rock	x	sp.
JKK11	E2	Nikko, Japan	rock	x	sp.
JKK12	E2	Nikko, Japan	rock	x	sp.
JVK1	E2	Nikko, Japan	rock	x	sp.
UW107	E2	Seattle, Washington, USA	rock	x	sp.
UW111	E2	Seattle, Washington, USA	rock	x	sp.
UW113	E2	Seattle, Washington, USA	rock	x	sp.
W319	E3	Talybont, Wales	rock	x	sp.
SAG 9.96	E3	Rijsenhout, Netherlands	above water level of alkaline lake	EU477438	fluitans
UO06	E3	Cleveland, Ohio, USA	rock	x	sp.
GALW015442	E3	Galway, Ireland	soil between tiles of pavement at the base of wall	EU477432	flaccidum
UW116	E3	Seattle, Washington, USA	rock	x	sp.
JKK15	E3	Nikko, Japan	rock	x	sp.
JVK4	E3	Nikko, Japan	rock	x	sp.
JVK6	E3	Nikko, Japan	rock	x	sp.
3KL_SOK	E3	Sokolov, Czech Republic	dump	x	sp.
ROS85/1	E4	Borchen, Germany	concreate	EU477426	sp.
GALW015468	E4	Copenhagen, Denmark	soil between tiles of pavement at the base of wall	EU477428	sp.
GALW015492	E4	Koper, Slovenia	concreate	EU477442	sp.
GALW015478	E4	London, Great Britain	concreate	EU477443	sp.
GALW015472	E4	Marseilles, France	concreate	EU477444	sp.
GALW015560	E4	Pavia, Italy	concreate	EU477448	sp.
GALW015300	E4	Pisa, Italy	concreate	EU477449	sp.
GALW015559	E4	Plymouth, Great Britain	concreate	EU477450	sp.

GALW015462	E4	Prague, Czech Republic	concreate	EU477452	sp.
GALW015298	E4	Siena, Italy	concreate	EU477453	sp.
GALW015493	E4	La Valletta, Malta	concreate	EU477455	sp.
W304	E4	Talybont, Wales	rock	x	sp.
W311	E4	Talybont, Wales	rock	x	sp.
W313	E4	Talybont, Wales	rock	x	sp.
W315	E4	Talybont, Wales	rock	x	sp.
W317	E4	Talybont, Wales	rock	x	sp.
UO09	E4	Cleveland, Ohio, USA	rock	x	sp.
UO17	E4	Cleveland, Ohio, USA	concreate	x	sp.
UO32	E4	Cleveland, Ohio, USA	concreate	x	sp.
UO37	E4	Cleveland, Ohio, USA	concreate	x	sp.
UO72	E4	Cleveland, Ohio, USA	rock	x	sp.
UO83	E4	Cleveland, Ohio, USA	bark	x	sp.
UO84	E4	Cleveland, Ohio, USA	bark	x	sp.
W320	E4	Talybont, Wales	rock	x	sp.
TR 18	E4	Australia	soil	HQ613257	sp.
UO02	E4	Cleveland, Ohio, USA	rock	x	sp.
SAG 2107	E4	x	x	HQ613254	sp.
SAG 2109	E4	x	x	HQ613256	sp.
W104	E4	Glynneath, Wales	rock	x	sp.
W210	E4	Brecon, Wales	rock	x	sp.
W212	E4	Brecon, Wales	rock	x	sp.
W215	E4	Brecon, Wales	rock	x	sp.
W218	E4	Brecon, Wales	rock	x	sp.
SAG 2065	E4	Roskilde, Denmark	in tree plantage	HQ613253	sp.
SAG 121.80	E5	Solling, Germany	bark of beech tree	EU477431	flaccidum

W209	E5	Brecon, Wales	rock	x	sp.
W211	E5	Brecon, Wales	rock	x	sp.
W217	E5	Brecon, Wales	rock	x	sp.
W219	E5	Brecon, Wales	rock	x	sp.
K09	E5	České středohoří Mts., Czech Republic	moss vegetation	HE649362	sp.
K40	E5	České středohoří Mts., Czech Republic	moss vegetation	HE649363	sp.
UNA00067477	E5	June lake, Florida, USA	wall of small wooden building	EU477437	sp.
UTEX 462	E6	Port Barrow, Alaska, USA	freshwater (snow)	AF408253	subtillissimum
SAG 384.1	E6	Port Barrow, Alaska, USA	freshwater (snow)	EU477454	subtillissimum
SAG 37.86	F	Bressanone, Italy	soil	HQ613241	crenulatum
UO05	F	Cleveland, Ohio, USA	rock	x	sp.
SAG 8.96	F	Valkenswaard, Netherlands	soil near water level of river Dommel	EU477445	mucosum
W109	F	Glynneath, Wales	rock	x	sp.
14613.5e	G	Koeboes, South Africa	biological soil crust	HQ613249	sp.
14621.6	G	Western Cape, South Africa	soil crust	HQ613250	sp.
LUK 318	G	Czech Republic	dumps after coal mining, from soil	HQ613251	sp.

3.3.Paper 3

Ryšánek D., Elster J., Kováčik L. and Škaloud P. (2016)
Diversity and dispersal capacities of a terrestrial algal genus *Klebsormidium* (Streptophyta) in polar regions.
FEMS Microbiology Ecology 92.4: fnw039.

Diversity and dispersal capacities of a terrestrial algal genus *Klebsormidium* (Streptophyta) in polar regions

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ABSTRACT

The distribution of microbial eukaryotes (protists) has been frequently discussed during the last two decades. The ubiquity hypothesis assumes the lack of latitudinal gradients in protist diversity due to their unlimited global dispersal. In this study, we examined the diversity and distribution of the very common, globally distributed green algal genus Klebsormidium across climatic zones, focusing on the polar regions. We tested whether (i) there is comparable diversity among the polar and temperate regions, and (ii) whether a spatial genetic differentiation occurs at the global scale. We collected a total of 58 Arctic, Antarctic and temperate strains, and genetically characterized them by sequencing the rbcL gene and two highly variable chloroplast markers. Our analyses revealed the presence of two different distribution patterns which are supposed to characterize both macroorganisms and protists. On the one hand, we demonstrated unlimited dispersal and intensive gene flow within one of the inferred lineages (superclade B). On the other hand, the majority of *Klebsormidium* clades showed rather a limited distribution. In addition, we detected a significant decrease of species richness towards the poles i.e. the macroecological pattern typical for macroorganisms. Species within a single protist genus may thus exhibit highly contrasting distribution patterns, based on their dispersal capabilities, which are usually shaped by both intrinsic and extrinsic factors.

INTRODUCTION

The distribution of microalgae is a major topic of modern microbial research (Caron 2009; Gast 2015). Two opposite hypotheses have been proposed: the ubiquity model (Finlay, Esteban and Fenchel 1996; Finlay 2002), which emphasizes the cosmopolitan distribution of protists; and the moderate endemicity model (Foissner 1999, 2006), which admits the existence of endemic species with limited distribution. Some authors proposed that the small organism size, large population sizes and high dispersal potential of eukaryotic microorganisms would lead to high gene flow across large geographical scales, resulting in an ubiquitous species distribution in suitable environments (Finlay 2002; Fenchel and Finlay 2004). Large population sizes would be expected to prevent local extinction and result in undisturbed population diversity (Fenchel and Finlay 2004), leading to high local genetic diversity (Mes 2008). In addition, intensive gene flow would constantly erase genetic diversity among populations, leading to a relatively low global diversity and undifferentiated populations (Fenchel and Finlay 2004).

Another consequence of the ubiquity hypothesis is that, as a result of global dispersal, latitudinal gradients in diversity should be weak or absent once ecological controls are factored out (Hillebrand and Azovsky 2001; Finlay and Fenchel 2004). However, there are only a few studies testing the presence of latitudinal gradients in eukaryotic microorganisms, moreover they are largely incongruent. Whereas Hillebrand and Azovsky (2001) showed that latitudinal gradients of species richness are largely absent for diatoms and presumably also for other unicellular and small multicellular organisms, the studies of Vyverman et al. (2007) and Siver and Lott (2012) showed these gradients on freshwater diatoms and silica-scaled chrysophytes, respectively. On the contrary, large organisms like plants and vertebrates show an obvious strong decrease of species richness towards the poles (see overview in Huston 1994). In addition, there should be significant differences in diversity between the poles due to the contiguous nature of the terrestrial Artic landmass with a temperate landmass at lower latitudes. Conversely, terrestrial Antarctica is a large isolated continent with small outlying sub-Antarctic islands. There, high polar ecosystems are biologically unique, with a more central role for bryophytes, lichens andmicrobial photoautotrophs over that of vascular plants. The biggest diversity differences between the poles are knownin vascular plants where 2218 species are recorded for the Arctic, but just two species for Antarctica (Pointing et al. 2015). Similar differences were also found in bryophytes and lichens, which constitute the major plant cover in the polar regions. Whereas ca 900 mosses and liverworts have been described from the Arctic (Walker et al. 2005), Antarctica hosts only ca 125 species covering a small

fraction of the total land area (Seppelt and Green 1998). For lichens, about 1750 species are known from the Artic, with 8%-10% of these species being endemic (Dahlberg and Bultmann 2013). On the contrary, ca 380 lichen species have been recorded in Antarctica (Øvstedal and Smith 2001), with about 21% of these being endemic taxa (Hertel 1988; Sancho et al. 1999).

Based on their cosmopolitan distribution and high dispersal, the diversity of microbial photoautotrophs should be comparable through the various regions (Pearce et al. 2009). Indeed, the estimated total number of species occurring in the Arctic was comparable to the diversity estimated for Antarctica (Pointing et al. 2015). However, this assumption has never been tested directly for whole microbial communities. Usually, the composition of polar microbial flora has been investigated separately either in Antarctica (e.g. Seaburg et al. 1979; Broady 1986, 1996; Pankow, Haendel and Richter 1991; Broady and Smith1994; Mataloni, Tell andWynn-Williams 2000; Cavacini 2001; Fermani, Mataloni and Van de Vijver 2007) or the Arctic region (Kaštovská et al. 2005, 2007; Stibal, Šabacká and Kaštovská 2006; Matula et al. 2007). Similarly, the currently available molecular data are very fragmentary, consisting of a number of isolated taxonomic and ecophysiological studies on individual taxa, such as various diatom species (Sabbe et al. 2003; Vyverman et al. 2010; Souffreau et al. 2013), green algae (Lesser et al. 2002; Pocock et al. 2004; Pichrtová et al. 2013; Pichrtová, Hájek and Elster 2014), xanthophytes (Broady, Ohtani and Ingerfeld 1997; Rybalka et al. 2009), ciliates (Petz et al. 2007), dinoflagellates (Montresor et al. 2003; Rengefors et al. 2008, 2015; Rengefors, Logares and Laybourn-Parry 2012) and lichen symbionts (Romeike et al. 2002; Fernández-Menroza et al. 2011; Domaschke et al. 2012). The majority of studies suggested a bipolar distribution of the investigated microautotrophs (Darling et al. 2000; Montresor et al. 2003; Fernández-Menroza et al. 2011; Domaschke et al. 2012). However, Petz et al. (2007) demonstrated that only 13% of ciliate species showed a bipolar distribution. Strunecký, Elster and Komárek (2010) even observed no similarities between the poles when investigating the diversity of the cyanobacterial genus *Phormidium*. In the most recent evaluation of protist diversity in the polar regions, Wolf et al. (2015) found a rather small overlap between the Arctic and Antarctica, ranging from 5.5% to 14.5% depending on the group investigated.

There is still a fruitful debate concerning the endemicity of protist organisms in polar regions. For example, identical cyanobacterial taxa have been reported from the Arctic, Antarctica and alpine lakes (Jungblut et al. 2005), while the existence of endemic species has been proposed within the cyanobacterial genus *Phormidium* (Strunecký, Elster and Komárek 2010). Some polar cyanobacteria occupying highly cryptic habitats, such as hypolithic substrates, may have been genetically isolated for an evolutionarily long time (Bahl et al.

2011). In diatoms, morphological studies suggested the existence of at least 40% endemic taxa in some Antarctic areas (Schmidt, Mäusbacher and Müller 1990; Sabbe et al. 2003). Currently, combined molecular, ecological and morphological studies have indicated far greater microbial endemism than previously assumed (Vyverman et al. 2010). Souffreau et al. (2013) presumed that cosmopolitan Antarctic diatom species are in fact species complexes, possibly containing Antarctic endemics with lowtemperature preferences. However, in contrast to diatoms and cyanobacteria, the green algal component ofmicrobial mats has remained virtually unstudied. The available data are largely restricted to morphological taxonomic inventories on the continent, such as Victoria Land (Cavacini 2001; Adams et al. 2006), the Antarctic Peninsula (Mataloni and Pose 2001) and maritime Antarctica (Fermani, Mataloni and Van de Vijver 2007; Zidarova 2007). Broady (1996) suggested that the vast majority of Antarctic terrestrial green algae are cosmopolitally distributed. However, this prediction has yet to be studied by modern molecular techniques.

In this study, we examined the diversity and distribution of the filamentous green algal genus *Klebsormidium* in the polar regions. The genus *Klebsormidium* is very common and diverse in temperate zones (Rindi and Guiry 2004; Rindi, Guiry and López-Bautista 2008; Rindi et al. 2011; Škaloud and Rindi 2013; Mikhailyuk et al. 2015; Ryšáanek, Hrčková and Škaloud 2015), but data about its occurrence in polar regions are still very scarce. The majority of its occurrence records comprises simple notes about their presence in various algal assemblages (Mataloni, Tell and Wynn-Williams 2000; Cavacini 2001; Kaštovská et al. 2005, 2007; Stibal, Šabacká and Kaštovská 2006; Fermani, Mataloni and Van de Vijver 2007; Matula et al. 2007).

The general aim of this study was to test whether there exists comparable diversity among the polar and temperate regions in green algal eukaryotic microorganisms. We used the genus *Klebsormidium* as a model evolutionary lineage of ubiquitous terrestrial protists. In addition, to test for the presence of spatial genetic differentiation at the global scale, we investigated the population structure of a selected globally distributed lineage by sequencing fast evolving cpDNA molecular markers.

MATERIALS AND METHODS

Sampling site and cultivation methods

During the period from 1989 to 2014, a total number of 12 expeditions were organized to investigate the diversity of algae and cyanobacteria in polar regions. Six expeditions were carried out at different Arctic and Antarctic regions, respectively, resulting in collecting over

500 samples in total (Table S1, Supporting Information). All samples were cultivated on Petri dishes on 1.5% agar supplemented with Bold's basal medium (BBM; Starr and Zeikus 1993). Detected *Klebsormidium* filaments were transferred to Petri dishes with fresh BBM medium. After three transfers, the obtained cultures were observed to be unialgal by examination under a light microscope. Samples and unialgal stock cultures of *Klebsormidium* were maintained in BBM at 15°C under white fluorescent illumination of 30–50 μmol photons m^{-2} s^{-1} provided by 18Wcool tubes (Philips TLD 18W/33, the Netherlands), with a light:dark (L:D) cycle of 14:10 h. For the purpose of the population structure analysis, an additional 26 temperate strains belonging to the superclade B sensu (Rindi et al. 2011) were isolated from limestone and basalt rocks in the Czech Republic and Slovakia (Table S2, Supporting Information).

Molecular analyses

A total of 32 *Klebsormidium* microcolonies (Table S2, Supporting Information) were used in subsequent molecular analyses. DNA was isolated according to the protocol published in Ryšáanek, Hrčková and Škaloud (2015), and stored at –20°C. The sequences of the rbcL gene, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, were obtained using polymerase chain reaction (PCR) amplification with a Touchgene Gradient cycler (Techne, UK). The rbcL gene was amplified using the primers KF590 (5´-GAT GAA AAC GTA AAC TCT CAG C-3´) and rbcL-KR2 (5´-GGT TGC CTT CGC GAG CTA-3´; Škaloud and Rindi 2013). Both primers were designed specifically to amplify *Klebsormidium* species. Each 20 μL reaction for PCR was conducted as described in Ryšánek, Hrčková and Škaloud (2015). The PCR protocol followed that of Škaloud and Rindi (2013). Sequencing reads were assembled and edited by using SeqAssem (Hepperle 2004).

For phylogenetic analyses, the newly obtained *Klebsormidium* rbcL sequences were added to the sequences available in the GenBank database to produce an alignment. The final alignment was constructed using ClustalW (Thompson, Higgins and Gibson 1994) with MEGA 5.05 (Tamura et al. 2011). The aligned data set was analysed by using Bayesian analysis (BI) with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001), maximum parsimony (MP) analysis with PAUP 4.0b10 (Swofford 2002), and maximum likelihood (ML) analysis with GARLI (Zwickl 2006). The evolutionary model was determined by using PAUP/MrModeltest 2.3 (Nylander 2004). The model selected under the Akaike Information Criterion was GTR + I + G. The BI analysis was performed using the prior set as the default in MrBayes; the robustness of the tree topologies was assessed by bootstrapping the data set as described by Škaloud and Rindi (2013).

Population structure analyses

A total of 51 *Klebsormidium* strains belonging to the superclade B sensu (Rindi et al. 2011) were subjected to the analysis of population structure (Table S1, Supporting Information). Two molecular markers were selected based on the analysis of recently published plastid genomes (Civáň et al. 2014; Hori et al. 2014), including the plastid spacers atpE-trnM and ndhK-ndhC. The sequenceswere obtained by using PCR amplification with a Touchgene Gradient cycler (Techne, UK). The spacer atpE-trnM was amplified by using the newly designed primers atpE F (5´-AGC ATT TCG TCG TGC CAA AGC A-3´) and trnM R (5´-GGT TCA AAT CCA AGT GCG ACC-3´). The spacer ndhK-ndhC was amplified by the newly designed primers ndhK F (5´-GTC CCA TAA AGC AAG GGC CA-3´) and ndhC R (5´-TGG AAT TGA GCC TGT GGG AG-3´). Each 20 µL reaction for PCR was conducted as described in Ryšánek, Hrčková and Škaloud (2015). PCR amplification of the spacer atpE-trnM began with an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C for 1.5 min, with a final extension at 72°C for 8min. The amplification of the spacer ndhK-ndhC began with an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 56°C for 1 min and elongation at 72°C for 1.5 min, with a final extension at 72°C for 8 min. The PCR products were quantified on a 1% agarose gel stained with ethidium bromide. The purification and sequencingwere performed as described in Škaloud and Rindi (2013). The sequencing reads were assembled and edited by using SeqAssem (Hepperle 2004). For illustrating the genetic diversity within the superclade B, we constructed the haplotype networks on the basis of ML analyses of the available sequences. The haplotype network was made in Haplotype Viewer (G. Ewing; available at www.cibiv.at/~greg/haploviewer).

RESULTS

Analysis of molecular diversity

A total of 32 strains were isolated from the polar regions, including 26 Arctic and six Antarctic strains. The overall diversity was relatively low in comparison to the genetic diversity identified in the temperate zone (Rindi et al. 2011; Škaloud and Rindi 2013; Ryšánek, Hrčková and Škaloud 2015). In general, our molecular investigations revealed the presence of eight genotypes belonging to four distinct *Klebsormidium* lineages (Fig. 1), identified as clades B, E1, E2 and E4 sensu (Rindi et al. 2011). The great majority of strains (77%) were inferred within clade B, consisting of both Arctic and Antarctic isolates. The

Arctic strain 818 inferred within clade E1 was related to strain K44, isolated from a peat bog in the Czech Republic (Škaloud and Rindi 2013). The three Arctic isolates belonging to clade E2 (ELS2, ELS3 and ELS4) were closely related to the members of lineage 4 sensu (Škaloud and Rindi 2013), consisting of aerophytic, synanthrophic strains isolated from Portugal, Germany, the Czech Republic and France. The remaining three strains inferred within clade E3 formed three separate genotypes. Whereas the two Antarctic strains LUC4 and LUC5 were related to the European aerophytic strains belonging to lineage 11 sensu (Škaloud and Rindi 2013), the Arctic strain 302 was inferred in the vicinity of the Australian terrestrial strain TR18. However, the relationship and exact phylogenetic position of the strains belonging to clade E2 remained unresolved by our analyses.

Population differentiation of the superclade B strains

To evaluate the intercontinental dispersal capabilities of the polar *Klebsormidium* strains, we conducted a population-level investigation of all available strains belonging to superclade B i.e. 21 Arctic, 4 Antarctic,and 26 temperate strains. The superclade B represents a well delimited species-level lineage exhibiting a very low genetic diversity among the investigated strains (Rindi et al. 2011, Škaloud et al. 2014, Ryšáanek, Hrčková and Škaloud 2015). Two highly variable, plastid-encoded spacers were used, including the 691 bp long spacer atpE-trnM (51 strains), and the 698 bp long spacer ndhK-ndhC (37 strains). Analyses of DNA variation in both sequenced spacers showed extensive sharing of haplotypes among the biomes, indicating the absence of geographical population structuring due to unlimited gene flow. A total of 23 different haplotypes were identified by analysis of the atpE-trnM sequences, of which 19were represented by a single sequence only (Fig. 2). The most frequent haplotype had 20 records, containing 17 temperate and three Arctic strains. The second most common haplotype had eight records, including one Antarctic and seven Arctic strains. The sequences of the spacer ndhK-ndhC were obtained for a total of 37 strains, including 20 Arctic,four Antarctic, and 13 temperate strains. A total of 19 different haplotypes were identified, of which eight were represented by a single sequence only (Fig. 3). Both of the two most common haplotypes (having four records each) were found in the two different biomes i.e. the Arctic-temperate and the Arctic-Antarctic regions. Haplotype sharing was detected in two additional cases (Fig. 3).

DISCUSSION

Diversity and abundance in polar regions

The genus *Klebsormidium* is one of the most abundant microautotrophs in various terrestrial and aerophytic habitats (Ettl and Gärtner 1995; Lokhorst 1996; John 2002, 2003). In fact, species of this genus are regularly listed as among the most abundant organisms found during diversity assessments of various habitat types worldwide (e.g. Lukešová and Hoffmann 1996; Neustupa 2001; Hoffmann, Ector and Kostikov 2007; Langhans, Storm and Schwabe 2009; Škaloud 2009; Schulz et al. 2015). Indeed, the recently published investigation of the *Klebsormidium* phylogeographic structure revealed its ubiquitous distribution on a global scale (Ryšánek, Hrčková and Škaloud 2015). All the above-mentioned studies thus imply the high global dispersal and comparable diversity estimates of the genus *Klebsormidium* through the various regions.

However, our investigation of newly isolated *Klebsormidium* strains revealed a conspicuously low genetic diversity in the polar regions as compared to the recently published DNA-based diversity assessments. Based on the molecular investigations of a number of isolated strains, Rindi et al. (2011) and Škaloud and Rindi (2013) delimited a total of 22 well-supported clades belonging to the seven major superclades A–G. In their evaluation of *Klebsormidium* diversity in Northern temperate mixed forests, Ryšánek, Hrčková and Škaloud (2015) found a total of 44 unique rbcL genotypes, indicating a very high genotypic diversity in the dataset based on 15 sampling sites only. Most recently, Mikhailyuk et al. (2015) detected more than 25 ITS rDNA genotypes from 16 different localities in alpine soil crusts.

In contrast to the previously mentioned investigations, we recovered a total of onlyeight rbcL genotypes. Such low genetic diversity could be partly explained by a relatively small number of investigated strains. However, the abundance of *Klebsormidium* in polar regions is obviously very low, which makes very hard to obtain a considerably greater amount of isolated strains. In fact, despite our extensive sampling effort in both the Arctic and Antarctica, only 32 strains were successfully isolated. Indeed, the total number of samples we investigated (over 500) greatly exceeded the number of sampling sites investigated by both Ryšánek, Hrčková and Škaloud (2015) and Mikhailyuk et al. (2015). We even failed to isolate a single *Klebsormidium* clone in several samples, despite repeated inoculation of the samples to Petri dishes (Table S1, Supporting Information). Instead, a high number of *Xanthonema* colonies were obtained, indicating the good preservation of algal communities but a very low, undetectable abundance of *Klebsormidium* species in these samples. This low abundance is in concordance with many studies which focused on terrestrial algal assemblages in both

Antarctica (Mataloni, Tell and Wynn-Williams 2000; Cavacini 2001; Fermani, Mataloni and Van de Vijver 2007) and the Arctic (Kaštovská et al. 2005, 2007; Stibal, Šabacká and Kaštovská 2006; Matula et al. 2007). In these studies, *Klebsormidium* was usually reported as a rare taxon, exceeded in abundance by other microautotrophs, such as *Leptolyngbya*, *Phormidium*, *Xanthonema* and *Chlorella*.

Despite the above-mentioned low global abundance of *Klebsormidium* in polar regions, we presume that the observed low genetic diversity can only partly be attributed to the effect of undersampling. Quite recently, Škaloud and Rindi (2013) investigated the ecological differentiation of *Klebsormidium* lineages based on the genetic characterization of a number of strains, including 27 newly isolated strains from the Czech Republic (central Europe). Although the area of the Czech Republic is incomparably smaller than that of polar regions, the genetic characterization of strains revealed the presence of 13 different genotypes belonging just into the single superclade E. Thus, using the comparable number of investigated strains (27 versus 32 strains), the diversity detected in a small temperate area significantly exceeds the total diversity found in both the Arctic and Antarctica (13 versus. 8 genotypes). We therefore suppose that the observed low genetic diversity might be rather attributable to the overall low abundance of *Klebsormidium* in polar regions (Vogt, Beisner and Prairie 2010).

Almost 80% of all polar *Klebsormidium* strains were inferred within the cosmopolitan superclade B sensu (Rindi et al. 2011). Interestingly, Mikhailyuk et al. (2015) reported this clade to grow in higher altitudes, near and above the pine-forest line in alpine regions. Such a distribution pattern, together with the resistance to both freezing and desiccation stresses reported for several superclade B strains (Elster et al. 2008), suggest either a strong adaptation of this lineage to polar environments or a preadaptation that developed in some strains, enabling them to participate in long-distance dispersal events, including to the poles.

Understanding the dispersal capacities

Considering its cosmopolitan distribution and predominance in the polar regions, superclade B represents an ideal model for testing the dispersal capabilities of microorganisms on a global scale. To differentiate the particular populations, we sequenced highly variable spacers between the chloroplast genes, a method frequently used in population structure assessment of higher plants (Doorduin et al. 2011; Hollingsworth, Graham and Little 2011). Themost common haplotypeswere shared across the arctic and temperate regions, indicating intensive gene flow and global dispersal. Such a high dispersal capacity explains

the lack of differences in eco-physiological performance of seven superclade B strains isolated from the Arctic (LUC9, LUC11 and LUC14), Antarctica (LUC6, LUC7 and LUC8) and the temperate zone (LUC2), as reported by Elster et al. (2008). Seemingly, the intensive gene flow at a global scale may prevent adaptation of populations to the local environment (Kawecki and Ebert 2004; Whitaker 2006). However, our knowledge about local adaptation mechanisms of protists is severely limited and needs further investigations (Weisse 2008; Weisse et al. 2011; Rengefors et al. 2015).

While this population genetic investigation shows clear evidence of a high dispersal capability of superclade B, the absence of several genotypes in the polar regions points to the restricted distribution of the majority of *Klebsormidium* lineages. Such a pattern supports the moderate endemicity model proposed by the contemporary protistologist (Foissner 1999, 2006; Gast 2015). Consequently, unlimited dispersal should be considerably limited in the majority of the lineages. Although filamentous, the great majority of *Klebsormidium* species easily disintegrate into fragments containing a few cells (Škaloud 2006). These can then spread because of random events, such as hurricanes or wind currents. Indeed, viable *Klebsormidium* cells have been detected in lower troposphere air samples (Overeem 1937; Sharma et al. 2007). Factors limiting dispersal should be then connected to airborne survival, which is mainly affected by UV radiation and desiccation (Isard and Gage 2001; Figuerola and Green 2002; Sharma et al. 2007).

Various physiological studies demonstrated that terrestrial algae have several mechanisms to provide protection and adaptation to high UV radiation, in particular by the accumulation of mycosporine-like amino acids (MAAs; Holzinger and Lütz 2006; Hughes 2006; Karsten, Lembcke and Schumann 2007; Pichrtová et al. 2013; Karsten and Holzinger 2014). However, strains belonging to superclade B had a lower content of MAAs in comparison to the other *Klebsormidium* lineages (Kitzing and Karsten 2015), excluding UV radiation as a crucial factor affecting the dispersal capabilities. On the other hand, desiccation intolerance seems to offer a promising explanation of the restricted distribution of several *Klebsormidium* lineages, in particular those of superclade E. This clade has been recognized as the most common worldwide, containing at least 14 distinct, mainly ubiquitous lineages (Škaloud and Rindi 2013; Mikhailyuk et al. 2015; Ryšánek, Hrčková and Škaloud 2015). However, the great majority of these lineages are absent in the polar regions. Evaluation of their distribution patterns and ecology indicated that these lineages are mainly restricted to humid and shaded habitats, or even to fresh waters (Škaloud and Rindi 2013; Mikhailyuk et al. 2015). Consequently, ecophysiological experiments did not reveal a high sensitivity to

desiccation stress in several strains belonging to superclade E (Karsten and Rindi 2010; Karsten and Holzinger 2012). We therefore hypothesize that dispersal capacities of particular *Klebsormidium* strains are mainly shaped by different adaptations to desiccation stress during their airborne transport.

Our investigation revealed that one of the most common members of terrestrial algal communities in temperate regions, the Streptophyte green algal genus *Klebsormidium*, exhibits two different distribution patterns common to both macroorganisms and protists. On the one hand, we demonstrated unlimited dispersal and intensive gene flow proposed to characterize the ubiquitous distribution of protists (Montresor et al. 2003; Petz et al. 2007). On the other hand, we showed a significant decrease of species richness towards the poles i.e. the distribution pattern typical for macroorganisms, such as higher plants and vertebrates (Huston 1994). Therefore, the proposed distinction between the distribution patterns of protists and macroorganisms (Hillebrand and Azovsky 2001; Fenchel and Finlay 2004) cannot be generalized to all organisms. In fact, even the species within a single genus may exhibit contrasting distribution patterns, based on their dispersal capacities, which are shaped by both intrinsic (e.g. adaptations to desiccation and UV) and extrinsic factors (e.g. the availability of suitable habitats).

ACKNOWLEDGEMENTS

The authors would like to thank Lenka Flašková for their kind support during molecular works.

FUNDING

This study was supported by a grant from the Charles University Science Foundation (GAUK no. 1544214). It was funded by the Ministry of Education, Youth and Sports of the Czech Republic projects: LM2010009, 7AMB14SK135 and the long-term research development project no. RVO67985939.

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FIGURES

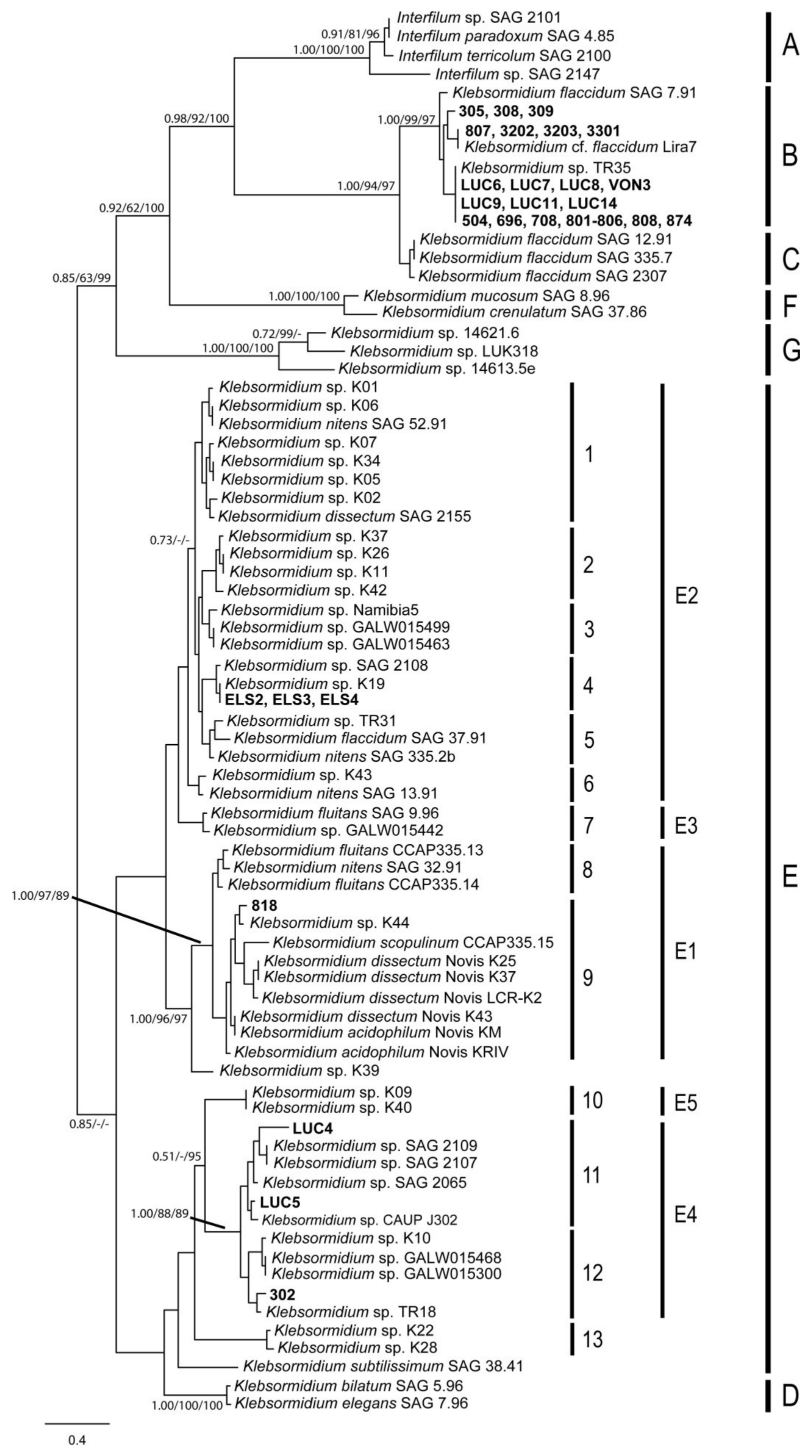
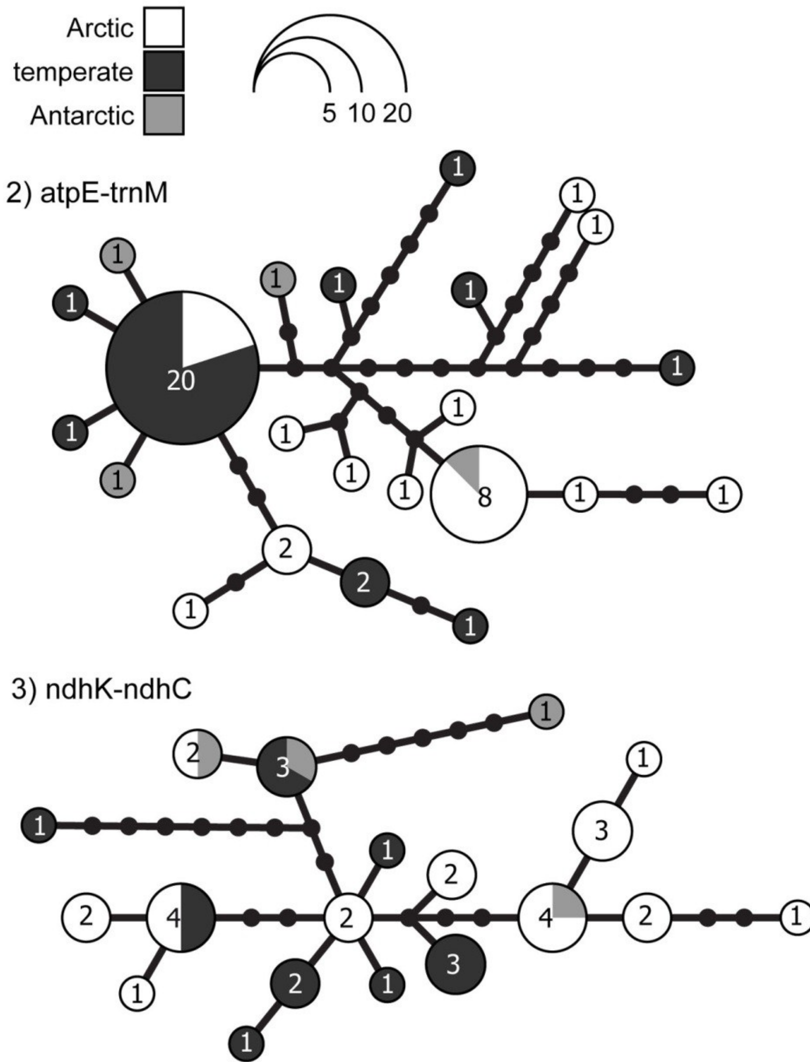


Figure 1. Phylogenetic tree obtained from BI based on an rbcL dataset, showing the position of the investigated *Klebsormidium* strains and their relatives. Values at the nodes indicate statistical support estimated by MrBayes posterior node probability (left), ML bootstrap (middle) and maximum parsimony bootstrap (right). The clade labelling (A–G, E1–E6) follows Rindi et al. (2011), the numbering of clades within the superclade E (1–13) follows Škaloud and Rindi (2013).



Figures 2 and 3. Haplotype genealogy from a ML tree of the spacer atpE-trnM (Fig. 2) and spacer ndhK-ndhC (Fig. 3), showing the relationship among haplotypes of the three regions. The circles represent the individual haplotypes. The scale shown on the upper left side of the figure indicates the relationship between circle sizes and the frequency of the haplotypes (numbers inside the circles specify the number of strains). Lines connecting the circles indicate a mutational step, and dots in the lines represent putative mutational steps between the haplotypes.

SUPPLEMENTARY DATA

Table 1.

		polar strains						
strains	**clade**	**authors**		**locality**	**coordinates**	***rbc*L**	**atpE-trnM**	**ndhK-ndhC**
818	E1	Snokhousova and Elster 2007/01	Arctic	Norther Sweeden, Lapland, Abisko, in vicinity of glacial moraine – Karsavagge, a small pool with mosses.	69°21'N, 18°49'E	Y		
ELS2	E2	Elster	Arctic	Svalbard, Mimerbukta, Pyramiden, shallow pool in centre of town	78°45' 55"N, 16°35' 45"E	Y		
ELS3	E2	Elster	Arctic	Svalbard, Adolfbukta, Kapp Napier, Brucebyen, shallow lake	78° 53' 52"N, 16° 52' 47"E	Y		
ELS4	E2	Elster	Arctic	Svalbard, Skansbukta, shallow pool close sea shore filled up by freshwater stream	78°52' 58"N, 16°04' 16"E	Y		
LUC4	E4	Jančušová 2004/01	Antarctica	Antarctica, King George Island, Admiralty Bay, Crepin Point, green sand near the bird nests	62°05´30.3"S, 58°28´32.3"W	Y		
LUC5	E4	Jančušová 2004/02	Antarctica	Antarctica, King George Island, Admiralty Bay, Crepin Point, green sand near the bird nests	62°05´30.3"S, 58°28´32.3"W	Y		
302	E4	Rysanek 2014/08	Arctic	Svalbard, sandstone	78°39'24.0"N, 16°18'57.7"E	Y		
305	B	Ryšánek 2014/08	Arctic	Svalbard, sandstone	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
308	B	Ryšánek 2014/08	Arctic	Svalbard, sandstone	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
309	B	Ryšánek 2014/08	Arctic	Svalbard, sandstone	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
504	B	Ryšánek 2014/08	Arctic	Svalbard, soil crust	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
696	B	Elster 1991/03	Arctic	Canada, Ellesmere island	79°08´N, 80°30´W	Y	Y	Y
708	B	Kastovska 2002/01	Arctic	Svalbard, Vestre Broeggerbreen, subglacial, river sediment	78°53´N, 11°80´E	Y	Y	Y

801	B	Ryšánek 2014/08	Arctic	Svalbard, coal	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
802	B	Ryšánek 2014/08	Arctic	Svalbard, coal	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
803	B	Ryšánek 2014/08	Arctic	Svalbard, coal	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
804	B	Ryšánek 2014/08	Arctic	Svalbard, coal	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
805	B	Ryšánek 2014/08	Arctic	Svalbard, coal	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
806	B	Ryšánek 2014/08	Arctic	Svalbard, coal	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
807	B	Ryšánek 2014/08	Arctic	Svalbard, coal	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
808	B	Ryšánek 2014/08	Arctic	Svalbard, coal	78°39'24.0"N, 16°18'57.7"E	Y	Y	Y
874	B	Snokhousova end Elster	Arctic	Svalbard, Hornsund, the foot of Angellfjellet, wet rocks with black biofilm.	77°00’N, 15°20’E	Y	Y	N
3202	B	Ryšánek 2014/08	Arctic	Svalbard, basalt	78°27'3.87"N, 16°20'4.53"E	Y	Y	Y
3203	B	Ryšánek 2014/08	Arctic	Svalbard, basalt	78°27'3.87"N, 16°20'4.53"E	Y	Y	Y
3301	B	Ryšánek 2014/08	Arctic	Svalbard, orthogneiss	78°43'04.6"N, 16°26'25.1"E	Y	Y	Y
LUC6	B	Kovacik 2003/08	Antarctica	Antarctica, King George Island, Keller Peninsula, green growth on remainder of whale bone near the seashore	62°05´02.9"S, 58°25´19.0"W	Y	Y	Y
LUC7	B	Kovacik 2003/08	Antarctica	Antarctica, King George Island, Keller Peninsula, green growth on remainder of whale bone near the seashore	62°05´02.9"S, 58°25´19.0"W	Y	Y	Y
LUC8	B	Kovacik 2003/03	Antarctica	Antarctica, King George Island, Keller Peninsula, green growth on the rocks near the seashore	62°03´48.5"S, 58°24´56.4"W	Y	Y	Y
LUC9	B	Elster 1991/03	Arctic	Canada, Ellsmere Island, produce dark-green mats in streaming water, these mats were most abundant in glacial front, central part of Sverdrup Pass, moraine area, Teardrop Glacier	79°08´N, 80°30´W	Y	Y	Y

LUC11	B	Kastovska 2002/04	Arctic	Svalbard, Ny-Alesund, Austre Broeggerbreen, cryoconite sediment	78°53´N, 11°80´E	Y	Y	Y
LUC14	B	Kastovska 2002/05	Arctic	Svalbard, Ny-Alesund, Vestre Broeggerbreen, cryoconite sediment	78°53´N, 11°80´E	Y	Y	Y
VON3	B	Snokhousova and Elster 2009	Antarctica	Antarctica, James Ross	64°13'S, 57°44'W	Y	Y	Y
		temperate strains						
C03	B	Ryšánek 2012/10	temperate	Czech Rep., České středohoří, basalt	50°35'39.16"N, 14° 5'54.11"E	Y	Y	N
DR01	B	Ryšánek 2015/03	temperate	Czech Rep., Prokopské udolí, limestone	50° 2'7.54"N, 14°21'2.10"E	Y	Y	Y
DR04	B	Ryšánek 2015/03	temperate	Czech Rep., Prokopské udolí, limestone	50° 2'7.54"N, 14°21'2.10"E	Y	Y	Y
J01	B	Ryšánek 2012/10	temperate	Czech Rep., Vápenná, limestone	50°16'38.67"N, 17° 5'19.90"E	Y	Y	Y
J05	B	Ryšánek 2012/10	temperate	Czech Rep., Vápenná, limestone	50°16'38.67"N, 17° 5'19.90"E	Y	Y	Y
J06	B	Ryšánek 2012/10	temperate	Czech Rep., Vápenná, limestone	50°16'38.67"N, 17° 5'19.90"E	Y	Y	Y
J07	B	Ryšánek 2012/10	temperate	Czech Rep., Vápenná, limestone	50°16'38.67"N, 17° 5'19.90"E	Y	Y	N
J08	B	Ryšánek 2012/10	temperate	Czech Rep., Vápenná, limestone	50°16'38.67"N, 17° 5'19.90"E	Y	Y	N
J11	B	Ryšánek 2012/10	temperate	Czech Rep., Vápenná, limestone	50°16'38.67"N, 17° 5'19.90"E	Y	Y	N
K01	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	N
K02	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	Y
K05	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	N
K06	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	N

K07	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	Y
K08	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	N
K09	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	N
K10	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	N
K12	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	N
K14	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	Y
K15	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	N
K20	B	Ryšánek 2011/05	temperate	Czech Rep., Český kras, limestone	49°56'49.96"N, 14° 5'25.49"E	Y	Y	N
LUC2	B	Kovacik 1998/13	temperate	Slovakia, Bratislava, subterranean jewish cemetery (crypt)	48°08´52.3"N, 17°05´46.4"E	Y	Y	Y
MA21	B	Ryšánek 2013/10	temperate	Czech Rep., Moravský kras, limestone	49°22'29.40"N, 16°43'36.78"E	Y	Y	Y
MA23	B	Ryšánek 2013/10	temperate	Czech Rep., Moravský kras, limestone	49°22'29.40"N, 16°43'36.78"E	Y	Y	Y
SK07	B	Ryšánek 2015/03	temperate	Czech Rep., Panská skála, basalt	50°46'50.94"N, 14°30'31.84"E	Y	Y	Y
SK11	B	Ryšánek 2015/03	temperate	Czech Rep., Panská skála, basalt	50°46'50.94"N, 14°30'31.84"E	Y	Y	Y

3.4. Paper 4

Ryšánek D., Holzinger A. and Škaloud P. (2016)
Influence of substrate and pH on the diversity of aeroterrestrial alga *Klebsormidium* (Klebsormidiales, Streptohyta): A potentially important factor for sympatric speciation.
Phycologia 55: 347-358.

Influence of substrate and pH on the diversity of the aeroterrestrial alga *Klebsormidium* (Klebsormidiales, Streptophyta): a potentially important factor for sympatric speciation

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ABSTRACT

Our knowledge of the processes involved in speciation of microalgae remains highly limited. In the present study, we investigated a potential role of ecological speciation processes in diversification of the filamentous green alga *Klebsormidium*. We examined 12 strains representing four different genotypes. The strains were collected from sandstone and limestone rocks and were cultivated at five different pH levels ranging from pH 4 to pH 8. We determined the responses of the 12 strains to the experimental pH conditions by (1) measuring the effective quantum yield of photosystem II, and (2) determining the growth rates after cultivation at different pH levels. Strong differences were found between the results obtained by these two methods. Direct counting of cells revealed a strong ecological differentiation of strains of Klebsormidium isolated from different substrate types. Strains isolated from limestone showed the highest growth rates at higher pH levels; whereas, the strains isolated from sandstone exhibited two distinct growth responses with optima at pH 5 and 6, respectively. In contrast, the effective quantum yield of photosystem II was always down-regulated at lower pH values, probably due to dissolved inorganic carbon limitation. In general, we determined distinct ecophysiological differentiation among distantly and closely related lineages, thereby corroborating our hypothesis that the sympatric speciation of terrestrial algae is driven by ecological divergence. We clearly showed that pH is a critical ecological factor that influences the diversity of autotrophic protists in terrestrial habitats.

INTRODUCTION

Protist species diversity has been the subject of considerable debate (Finlay 2002; Foissner 2006; Caron 2009). On one hand, some researchers think that diversity is very low because protists show a cosmopolitan distribution without endemic species. This ubiquity model predicts a very low probability of local extinction within protist populations (Fenchel & Finlay 2003). The consequence of this very low local extinction, coupled with extremely large population size, is high local protist diversity and low global diversity (Fenchel & Finlay 2003, 2004). Supporters of the ubiquity model estimate that approximately 20,000 protist species exist (Finlay & Fenchel 1999). Fenchel & Finlay (2003) argued that the distribution of the smallest organisms is dependent solely on habitat properties and not on contingencies of the evolutionary history, as is the case for multicellular organisms. On the other hand, other researchers think that diversity is very high because microorganisms also have a significant portion of endemic species. The existence of endemic species has been demonstrated through the example of flagship species, i.e. species with easily recognizable morphologies for which presence/absence can readily be demonstrated (Foissner 2006; Foissner et al. 2008). The considerable protist diversity is puzzling because the current paradigm holds that dispersal in microbes is ubiquitous, and therefore allopatric speciation must be strongly impeded by global gene flow. However, virtually nothing is known about alternative speciation mechanisms. Allopatric processes may play an important role in diatom speciation (Evans et al. 2009; Casteleyn et al. 2010). Moreover, some studies have demonstrated sympatric speciation (reviewed in Benton & Pearson 2001) based on fossil evidence. Nevertheless, it is clear that the distribution of some protists on the Earth's surface is restricted by their habitat requirements. In addition, convergent morphological evolution has frequently led to the existence of simple morphotypes, which show extremely high phylogenetic diversity (Huss et al. 1999). Recently, many molecular studies have demonstrated the existence of very high cryptic diversity in different protistan taxa (de Vargas et al. 1999; von der Heyden et al. 2004; Škaloud & Peksa 2010; Škaloud et al. 2012).

Ecological adaptation is also known to influence diversity in algae (Huss et al. 2002; Logares et al. 2007; Rindi et al. 2008; Škaloud & Rindi 2013; Škaloud et al. 2014). For example, Rindi and Guiry (2002) reported that some species of *Trentepohlia* and *Printzina* formed perennial populations on different substrates in urban habitats in western Ireland. Similar ecological differentiation was reported for some species of *Prasiola* (Trebouxiophyceae) (Moniz et al. 2012). The influence of environmental factors was further demonstrated by Peksa & Škaloud (2011), who showed that specific lineages within the

lichen photobiont genus *Asterochloris* exhibited clear environmental preferences, in relation to factors such as exposure to rain or sunlight, substrate type, and climate.

In comparison with the factors that determine the diversity of marine and freshwater algal communities (Machová -Černá & Neustupa 2009; Desrosiers et al. 2013; Svoboda et al. 2014), the critical factors that influence the distribution of aeroterrestrial algae remain unclear. The key factors that influence the community structure of terrestrial algae include light, humidity, temperature, nutrients, and pH (Hoffmann 1989). Pietrasiak et al. (2011) showed that in the absence of disturbance, several abiotic factors, particularly soil texture, pH, and electrical conductivity, seemed to be important for the development of biotic crusts. The pH seems to influence the dominance of the major groups of soil photoautotrophic organisms; cyanobacteria are known to prefer neutral and alkaline soils (Shields & Durell 1964; Brock 1973); whereas, green algae predominantly prefer acidic soils (Starks et al. 1981; Lukešová & Hoffmann 1995; Lukešová 2001). Bates et al. (2012) think that soil protistan richness and diversity were primarily influenced by climatic conditions that regulate annual moisture availability in soils (Bates et al. 2012). Similarly, climatic factors probably control the species composition of aerophytic algal communities growing on urban walls, e.g. Prasiolales-dominated assemblages in Atlantic parts of Europe vs *Klebsormidium*-dominated growths in continental and Mediterranean cities (Rindi & Guiry 2004; Rindi et al. 2007). Algal communities isolated from sandstone that was used as building blocks for a German castle differed markedly according to their exposure to direct sunlight (Hallmann et al. 2013).

In the present study, we investigated the genus *Klebsormidium* (Silva et al. 1972; Sluiman et al. 2008; Rindi et al. 2011) as a model to obtain a better insight into the ecologically driven speciation of microalgae. This genus comprises cosmopolitan filamentous green algae broadly distributed in various terrestrial and freshwater habitats (Rindi et al. 2008, 2011; Škaloud & Rindi 2013; Mikhailyuk et al. 2014, 2015; Ryšánek et al. 2015). The local distribution of *Klebsormidium* is generally influenced by different substrate preferences (Novis 2006; Rindi et al. 2008, 2011; Škaloud & Rindi 2013; Škaloud et al. 2014), which repeatedly originated during the evolution of the genus (Škaloud et al. 2014). Strains of *Klebsormidium* show a wide range of adaptation to unfavourable conditions, such as prolonged desiccation (Karsten & Holzinger 2012; Karsten et al. 2013; Holzinger et al. 2014; Herburger & Holzinger 2015; Herburger et al. 2016; Karsten et al. 2016), low temperature (Elster et al. 2008; Nagao et al. 2008), heavy metals (Gaysina et al. 2009), and ultraviolet (UV) radiation (Nagao et al. 2008; Kitzing et al. 2014).

The main objective of the present study was to understand whether ecological preferences may be drivers of sympatric speciation in *Klebsormidium*.We focused on pH as one of the major factors influencing the diversity of terrestrial photoautotrophs (see above) and investigated its influence on the effective quantum yield and growth rate of several strains of *Klebsormidium* isolated from two different substrates, sandstone and limestone. We posed the following questions: (1) Do strains isolated from different substrates show differences in their response to pH? (2) Do different genotypes that inhabit the same substrate show differences in their physiological and growth responses? (3) Do closely related strains isolated from different substrates show differences in their response to pH?

MATERIAL AND METHODS

Sampling sites and cultivation methods

We collected and isolated algal samples from rocks in the Czech Republic during the autumn seasons of 2012 and 2013. The strains isolated from sandstone (P05, P08, P09, AD31, AD32, and AD36) were collected in Labské pískovce (P05, P08, P09) (50°48´N, 14°14´E) and Adršpach (AD31, AD32, AD36) (50°36´N, 16°70´E). The strains isolated from limestone (J06, J07, J11, MA12, MA16, and MA24) were collected near the village of Vápenná (J06, J07, J11) (50°16´N, 17°5´E) and in Moravský kras (MA12, MA16, MA24) (49°22´N, 16°43´E). The pH levels of the sandstone substrata from Labské pískovce and Adršpach were 5.05 and 5.17, respectively. The pH levels of the limestone substrata from Vápenná and Moravský kras were 6.97 and 7.06, respectively. The pH was measured by WTW pH-330 set with a flathead electrode (WTW SenTix Sur, Weilheim, Germany). The pH was measured on 10 different sides of rock, at each locality. All strains of *Klebsormidium* were isolated by cultivating samples from rock on 1.5% agar supplemented with Bold's basal medium (BBM) (Starr & Zeikus 1993). The selected algal filaments were transferred repeatedly to fresh Petri dishes. After three changes of each isolate to fresh Petri dishes, the obtained cultures were observed to be unialgal by examination under an Olympus CX 31 light microscope (Olympus Corp., Tokyo, Japan). Unialgal stock cultures of *Klebsormidium* were maintained in BBM at 20°C under white fluorescent illumination of 30–50 μmol photons m^{-2} s^{-1} provided by 18 W cool tubes (Philips TLD 18W/33, Amsterdam, the Netherlands), with a light:dark (L:D) cycle of 14:10 hours. We examined the morphology of 5-week-old cultures during the exponential growth phase.

Molecular analyses

The DNA was isolated according to the protocol of Ryšánek et al. (2015) and stored at -20°C. We used 12 microcolonies of *Klebsormidium* for subsequent molecular analyses. For molecular screening of the isolated strains we used partial sequences of the plastid-encoded rbcL gene (the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase). The *rbc*L sequences were obtained by polymerase chain reaction (PCR) with a Touchgene Gradient cycler (Techne, Cambridge, United Kingdom) using the primers KF590 (5´-GAT GAA AAC GTA AAC TCT CAG C-3´) and *rbc*L-KR2 (5´-GGT TGC CTT CGC GAG CTA-30; Škaloud & Rindi 2013). Both primers were designed specifically to amplify species of *Klebsormidium*. Each 20 μl reaction for PCR was composed as described by Ryšánek et al. (2015). The PCR protocol followed that of Škaloud & Rindi (2013). Sequencing reads were assembled and edited by using the SeqAssem software (Hepperle 2004).

For phylogenetic analyses, we used the newly obtained rbcL sequences of Klebsormidium and a selection of *rbc*L sequences of Klebsormidiales available in GenBank to produce an alignment. The final alignment of 632 base pairs (bp) was constructed by using ClustalW (Thompson et al. 1994) with MEGA 5.05 (Tamura et al. 2011). The aligned data set was analysed by using maximum parsimony with Phylogenetic Analysis Using Parsimony (PAUP 4.0b10; Swofford 2002), maximum likelihood with the Genetic Algorithm for Rapid Likelihood Inference (Zwickl 2006, unpublished Ph.D. dissertation), and Bayesian inference (BI) analysis with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). The evolutionary model was determined by using PAUP/MrModeltest 2.3 (Nylander 2004). The model selected under the Akaike information criterion was GTR + I + G. The BI analysis was performed by using the priors set as default in MrBayes. The robustness of the tree topologies was assessed by bootstrapping the data set as described by Škaloud & Rindi (2013).

Effective quantum yield in liquid medium

Six strains of *Klebsormidium* (limestone strains J06, J07, J11 and sandstone strains P05, P08, P09) were selected to evaluate their physiological performances in liquid medium. The exponentially growing strains were inoculated into 50-ml Erlenmeyer flasks containing fresh BBM medium. The strains were grown at five different pH levels (pH 4, pH 5, pH 6, pH 7, and pH 8). Liquid BBM medium was buffered to pH 4, pH 5, or pH 6 with 1 mM 2-(*N*-morpholino) ethanesulphonic acid and to pH 7 or pH 8 with 1 mM *N*-(2-hydroxyethyl) piperazine-*N*´-2-ethanesulfonic acid hemisodium salt. The pH was checked and adjusted at 3 day intervals by using 0.1 M NaOH or 0.1 M HCl (InoLab pH/ conductometer 720, WTW).

The strains were cultivated at an optimum growth temperature of 20°C under continuous white fluorescent illumination of 20 μmol photons m^{-2} $second^{-1}$ for 8 days. Cultures of *Klebsormidium* (each with an approximate volume of 100 μl four times) were harvested daily and were concentrated on Whatman GF/F glass fibre filters (Whatman, Seattle, Washington USA). The filters were saturated with BBM at a similar pH as in harvested bottles and were maintained for 2 hours in Petri dishes at ambient room temperature (~22°C) under continuous

white fluorescent illumination of 20–25 μmol photons m^{-2} s^{-1}. The effective quantum yield ($\Delta F/F_m'$) of photochemistry was determined at regular intervals by using a pulse-amplitude modulated fluorimeter (PAM 2500; Heinz Walz GmbH, Pfullingen, Germany). The physiological state of the photosynthetic apparatus was determined by measuring the effective quantum yield of photosystem II photochemistry in a dark-acclimated state ($\Delta F/F_m'$). The

measurements were made after 2 and 3 hours, with four replicates per strain. In total, we performed eight measurements for each pH level.

Effective quantum yield on rock substrate

Eight strains of *Klebsormidium* (limestone strains MA12, MA24, J06, J07 and sandstone strains AD31, AD32, AD36, P05) were selected to investigate their performances when growing directly on rocky substrata. Small pieces of rock (c. 1 cm in diameter) were transferred to Petri dishes and were stabilized by using 1.5% solidified agar (in distilled water). Each strain was cultivated on sandstone (n = 4) and limestone (n = 4) rocks (Fig. S1) at an optimum growth temperature of 20°C under continuous white fluorescent illumination of ~20 μmol photons m^{-2} $second^{-1}$ for 10 days. The effective quantum yield ($\Delta F/F_m'$) of photochemistry was determined daily by using a pulse-amplitude modulated fluorimeter (PAM 2500; Heinz Walz GmbH), with four replicates per strain.

Growth rate estimations

All 12 cultivated strains were used for growth rate measurements. After 2–3 weeks, approximately 1–1.5 ml of the experimental cultures growing in 50-ml Erlenmeyer flasks with fresh BBM medium were harvested into 2-ml tubes (Eppendorf, Hamburg, Germany) containing 2–3 glass balls, each with a diameter of 0.5 mm (Sigma-Aldrich, St Louis, Missouri USA). The tubes were inserted into a mill for grinding plant material (Retsch MM400, Haan, Germany) to fragment filaments into single cells; the mill was operated for 2–3 minutes at 18–24 frequencies/second. Next, approximately 80–120 μl of the solution

(containing filament fragments of differing sizes) were pipetted onto Petri dishes (diameter 8 cm). To monitor the subsequent growth of single-cell fragments, we pipetted very low cell densities (c. 2600 cell fragments/ml). All strains were grown at different pH levels (pH 4, pH 5, pH 6, pH 7, and pH 8) in Petri dishes containing 1.5% agar supplemented with BBM. The solidified BBM medium was buffered in the same manner as described above for the experiment in liquid medium. The pH was measured at 2-day intervals by using a WTW 330/SET1 with SenTix Sur electrode. Strains were cultivated at an optimum growth temperature of 20°C under continuous white fluorescent illumination of 20 μmol photons m^{-2} $second^{-1}$ for 4 days. The culture time was relatively short because the pH increased slowly, and we were unable to adjust the pH of the agar plates. At the start of the experiment, we selected approximately 30–40 single cells for cultivation at each pH level, and we subsequently monitored the growth of each single cell over four consecutive days. Each day, we recorded the length of the filaments (number of cells) grown from these single cells; we used these measurements to determine the growth rates based on 30–40 replicates.We counted the cells by direct observation using an Olympus CX 31 light microscope.

Data analysis

The effect of different pH values and rock surfaces on effective quantum yield ($\Delta F/F_m'$) was evaluated by multisample nonparametric Friedman two-way analysis of variance (ANOVA) tests by ranks, in connection with the Statistica software (StatSoft Inc, Tulsa, Oklahoma USA). Post hoc nonparametric multiple comparisons were computed by applying the '*Post Hoc* For Friedman.svb' macro, comparing the absolute values of the differences for all analyzed pairs. Pair differences in mean ranks were displayed in R (R Core Team 2016), using the package corrplot. Growth response to different pH values was tested by one-way ANOVA tests with the Tukey's pairwise comparisons in the program PAST 2.17c (Hammer et al. 2001). The graphs were created in SigmaPlot. The signifikance was tested to three levels: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

RESULTS

Strain morphology and molecular analyses

The results of our molecular analyses revealed that the isolated strains were representative of four different genotypes, which were specific to the sampled localities (Fig. 1). These four genotypes were inferred within three distinct clades. Two genotypes (sandstone strains P05, P08, and P09 and limestone strains MA12, MA16, and MA24) were inferred

within clade E1 sensu Škaloud & Rindi (2013); sandstone strains AD31, AD32, and AD36 were inferred within clade E13 sensu Škaloud & Rindi (2013); and limestone strains J06, J07, and J11 were shown to belong to superclade B sensu Rindi et al. (2011). The rbcL sequences of the 12 investigated strains of *Klebsormidium* were deposited in GenBank under accession numbers KU528666–KU528677. With the exception of superclade B, in which small differences between strains were observed, the strain morphology was relatively uniform within and among clades. In most of the strains, the filaments were short, 4–6 μm wide, and tended to segregate into shorter filaments or individual cells. Cells were 1.4–3.2 times longer than wide; the chloroplast covered 50% of the cell wall and usually possessed a small pyrenoid. With the exception of strain J11, H-shaped pieces were not observed.

Response to pH

EFFECTIVE QUANTUM YIELD IN LIQUID MEDIUM: In our first approach to evaluate the organisms' response to different pH values, we determined the physiological performance of three limestone strains (J06, J07, and J11) and three sandstone strains (P05, P08, and P09) in liquid media buffered to distinct pH values. We used a pulse-amplitude modulated fluorimeter to measure the effective quantum yield of photosystem II (Figs 2–7; Tables S1–S2). In all investigated strains, the effective quantum yield ($\Delta F/F_m'$) showed different responses to pH: $\Delta F/F_m'$ was significantly higher at pH 8 than at lower pH values (Figs 8–13), as shown by the two-way Friedman ANOVA tests, with the single exception of strain P09 (Fig. 13). In the strain P08 (Fig. 12), the effective quantum yield was significantly higher at pH 4 than at pH 6, as well. Throughout the experiments, the lowest $\Delta F/F_m'$ values were measured at pH 5 (Fig. 8).

EFFECTIVE QUANTUM YIELD ON ROCK SUBSTRATE: In our second approach, we cultivated four sandstone (AD31, AD32, AD36, and P05) and four limestone (MA12, MA24, J06, and J07) strains, respectively, directly on pieces of sandstone and limestone. As with the previous experiments, the physiological performance of investigated strains was evaluated by measuring the effective quantum yield of photosystem II ($\Delta F/F_m'$). We found no significant differences in physiological performances of limestone and sandstone strains when they were cultivated on the same type of rock (Figs 14–15; Tables S3–S4). However, Friedman two-way ANOVA tests revealed significant differences in the pairwise comparisons of the physiological performance of some strains (Fig. 16). $\Delta F/F_m'$ was significantly higher on

the strains cultivated on limestone, as determined by two-way Friedman ANOVA tests. Interestingly, the $\Delta F/F_m'$ of the limestone strains were inhibited during the first 4 days of cultivation on sandstone (Fig. 14). After this acclimation period, their physiological performance was, however, comparable to the sandstone strains.

GROWTH RATE ESTIMATIONS: Finally, we evaluated the influence of pH on the specific growth rates of all 12 cultivated strains of *Klebsormidium*.We found that all strains isolated from limestone (MA12, MA16, MA24, J06, J07, and J11) exhibited similar responses to pH; these strains showed increases in growth rate as the pH of the medium increased (Figs 17–18). After 4 days of cultivation, we identically counted only three cells at pH 4 but .10 cells at pH 7 and pH 8. However, strains J06, J11, MA16, and MA24 showed the highest growth rates at pH 7; whereas, strains J07 and MA12 showed the highest growth rates at pH 8. Strains MA12, MA16, and MA24 grew slightly faster than did strains J06, J07, and J11. The statistical tests revealed no significant differences in growth rates between pH 7 and 8 but the growth rates at other pH values were significantly different ($P < 0.001$), as determined by one-way ANOVA tests.

We observed different responses to pH between the two sandstone genotypes (Figs 19–20). Strains belonging to clade E13 (AD31, AD32, and AD36; Fig. 19) showed significantly different growth patterns ($P < 0.001$) at the investigated pH levels. These strains exhibited the highest growth rates at pH 6; at this pH, they produced an average of 10 cells after 4 days of cultivation. In contrast, at pH 4 and pH 8, these strains grew more slowly and produced only approximately two cells after 4 days. Strains belonging to clade E1 (Fig. 20) grew well at all investigated pH levels; however, they showed higher growth rates at pH 5 than at pH 8 (c. 9 cells vs c. 4–6 cells). For E1 sandstone strains, the statistical tests supported the significant differentiation of growth rates at pH 5 and 8, respectively ($P < 0.001$). The highest growth rate of E13 sandstone strains at pH 6 was also significantly supported ($P < 0.001$).

DISCUSSION

In this study, we applied two different methods to monitor the response of strains of *Klebsormidium* to different pH levels. First, we used a pulse-amplitude modulated fluorimeter to measure the effective quantum yield ($\Delta F/F_m'$) of photosystem II. The main advantage of this method is that it enables the maintenance of cultures in liquid medium, thereby allowing pH adjustment during cultivation. This in turn enables experiments to be continued for

prolonged periods (up to 8 days in the present study). Second, we used direct counting of cells on agar plates. This method is straightforward and provides an accurate estimate of cell growth rates at different pH levels. However, it is almost impossible to adjust the pH during the experiment. After 4 days in the present study, the pH had increased by c. 0.5 pH units. This increase in pH during cultivation experiments is caused by the photosynthetic activity of phototrophic organisms (Shiraiwa et al. 1993). Therefore, this technique should be used only for algal strains with relatively high growth rates.

Interestingly, we found clear differences between the results obtained by these two methods. Direct counting of cells revealed a strong ecological differentiation of strains of *Klebsormidium* isolated from different substrate types. Strains isolated from limestone showed the highest growth rates at pH 7 and pH 8 and had significantly lower growth rates at pH 4 and pH 5. The strains isolated from sandstone exhibited distinct growth responses. While strains belonging to clade E1 showed generally similar growth rates at all investigated pH levels, the strains inferred within clade E13 showed the highest growth rate at pH 6.

In contrast, the effective quantum yield ($\Delta F/F_m'$) of photosystem II did not show any differentiation between the strains isolated from different rock types. Instead, all investigated strains showed the highest $\Delta F/F_m'$ at pH 8, and its down-regulation at lower pH values. Such coincident responses indicate the presence of a common mechanism inducing an increase of photosynthetic efficiency at higher pH values.

Since the intracellular pH of photoautotrophs is usually maintained fairly constant over a wide range of external pH values (Lane & Burris 1981), we hypothesize that a strong positive effect of pH to $\Delta F/F_m'$ was probably caused by dissolved inorganic carbon (DIC) limitation at lower pH values. In aqueous solutions, the dissolved CO2 dissociates into bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}), maintaining a certain ratio depending on pH, ion concentrations, and salinity (Falkowski & Raven 2007). At high pH values, HCO_3^- is the dominant carbon species. However, Rubisco reacts only with dCO2, not bicarbonate or carbonate ions (Baba & Shiraiwa 2012). The majority of microbial phototrophs use dCO2 when it is freely available (Reynolds 1984). However, some Cyanobacteria and green algae use both dCO2 and HCO_3^- in photosynthesis due to the extra- and intracellular activity of carbonic anhydrase (Allen & Spence 1981). Although the use of different DIC components has not been studied in *Klebsormidium*, we consider the stimulation of photosynthesis by HCO_3^- as the most likely explanation of the positive correlation of $\Delta F/F_m'$ and pH in investigated strains (Shelp & Canvin 1980). In general, our results clearly show that, at least

in our experimental system, $\Delta F/F_m'$ does not reflect the overal fitness of studied organisms but rather indicates the efficiency of PSII under different DIC conditions. We therefore consider growth rate responses to different pH as the only measured data useful for assessing the ecological adaptation of the studied strains.

The tolerance of the investigated strains of *Klebsormidium* to a wide range of pH levels is in accordance with the results of previous investigations of the ecophysiology of this genus (Karsten et al. 2014). In general, strains of *Klebsormidium* are relatively tolerant to various physiological stresses such as UV radiation (Nagao et al. 2008; Kitzing et al. 2014; Kitzing & Karsten 2015), desiccation (Karsten et al. 2010, 2016; Karsten & Holzinger 2014), and osmotic stress (Kaplan et al. 2012). The genomic machinery required for adaptation to terrestrial environments was detected in the genome of *Klebsormidium flaccidum* (Kützing) P.C.Silva, K.R.Mattox & W.H.Blackwell (Hori et al. 2014), which is a member of clade E5 sensu Škaloud & Rindi (2013). This machinery includes genes involved in the signalling pathways for the phytohormones cytokinin, ABA, and ethylene, which was recently confirmed in the desiccation transcriptome of Klebsormidium crenulatum (Holzinger & Becker 2015). This is interesting, as the receptors of, e.g. cytokinin signalling show a strictly pH-dependent ligand binding in vascular plants (Lomin et al. 2015).

This ability to adapt to a wide range of conditions typical of terrestrial environments may explain the cosmopolitan distribution of the genus *Klebsormidium* (Ryšánek et al. 2015). Nevertheless, the relatively high abundance of this genus in different types of localities might simply be a sampling artefact. In other words, strains that are common and geographically widespread have been discovered and studied; whereas, rarer strains (e.g. such as those belonging to clade E13) have either not yet been studied or have been studied only sporadically. In the present study, strains belonging to clade E13 grew optimally in a very narrow range of pH levels; they showed the highest growth rates at pH 6 but grew very slowly at pH 4 and pH 8. This finding might imply that clade E13 is a specialist rather than a generalist, and this in turn may reflect its ecologically restricted occurrence. Generalists have a wider range of suitable habitats and are therefore discovered more frequently; in addition, they are geographically more widespread than are specialists (Finlay et al. 2002).

In the present study, we showed clear differences in growth responses between strains isolated from sandstone (lower pH preference) and limestone (higher pH preference) substrates. Our results are similar to those of Lowe et al. (2007), who reported a relatively important influence of pH on the structure of an algal community growing on a wet wall. Indeed, acidic conditions strongly increase the chemical solubility, and thus mobility of

metals, resulting in high concentrations of heavy metals such as Fe, Cu, Pb, Al, and Zn in soils and waters with low pH levels (Gross 2000; Aguilera et al. 2007; Novis & Harding 2007). High concentrations of heavy metals can therefore substantially influence the diversity of terrestrial microalgae growing on acidic substrates.

Our results suggest that different lineages of *Klebsormidium* are adapted to the substrate on which they originally occur, independently of their evolutionary distance. We found that closely related lineages differed ecophysiologically to the same extent as unrelated clades. Our findings may indicate the widespread existence of sympatric speciation in *Klebsormidium* through ecological divergence, and we hypothesize that this situation is probably common among other taxa of terrestrial algae. The mechanisms of genetic differentiation are not yet fully understood. Specializations to habitats (Gächter & Weisse 2006; Logares et al. 2007), selection pressures (Vanormelingen et al. 2009), and/or persistent founder effects (De Meester et al. 2002) have been hypothesized as important factors contributing to the structure of protist populations. Ecological differentiation facilitates allopatric (our data; de Vargas et al. 1999) and sympatric (e.g. Amato et al. 2007; Weisse 2008; Vanelslander et al. 2009) speciation of protistan cryptic species. Congruent with the recent studies of Fontaneto et al. (2007) and Birky et al. (2010), our data suggest the existence of distinct species units and sympatric speciation in asexual protists.

In addition, we found that adaption to specific substrates has originated many times during the evolutionary history of *Klebsormidium*. Strains belonging to clade E13 grew optimally in a very narrow range of pH levels. Moreover, two closely related genotypes inferred within clade E1 showed clear ecophysiological adaptation to the substrate from which they were originally sampled. These genotypes differed in their ecology and ecophysiology but were genetically similar. An analogous situation was reported by Logares et al. (2007), who investigated two dinophytes that had identical ribosomal DNAs but which differed from each other ecologically, physiologically, and even phenetically. Such clear differentiation has been attributed to rapid adaptive evolution, which has not yet been reflected in the ribosomal divergence.

In summary, in the present study, we showed that all strains isolated from sandstone and limestone were able to grow over the range of investigated pH levels but to differing extents. Strains isolated from limestone showed the highest growth rates at pH 7 and pH 8; these strains grew very slowly at pH 4 and pH 5. Strains isolated from sandstone exhibited two different growth responses. Strains from one of the investigated genotypes showed the highest growth rate at pH 6; whereas, strains of the other genotype had almost identical

growth rates at all of the investigated pH levels. We conclude that pH is a critical ecological factor that influences the diversity of *Klebsormidium* in terrestrial habitats. Moreover, our data highlighted distinct ecophysiological differentiation among distantly and closely related lineages, thereby corroborating our hypothesis that the common sympatric speciation of terrestrial algae is driven by ecological divergence. However, further research will be necessary to provide support to this general conclusion.

ACKNOWLEDGEMENTS

We would like to thank Prof. Ulf Karsten, University of Rostock, Germany, for several helpful suggestions and discussion on the experimental design of the study. This study was supported by AKTION 'Austria-Czech Republic' (http://www.oead.at), Project 65 p5 to Martina Pichrtová and A.H., Charles University Prague, and by a grant from the Charles University Science Foundation (GAUK n. 1544214). Moreover the study was supported by the Austrian Science Fund (FWF) Project P 24242-B16 and FWF project I 1951-B16 to AH and by The Czech Science Foundation grant 15-34645 L to Martina Pichrtová, Charles University, Prague.

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FIGURES

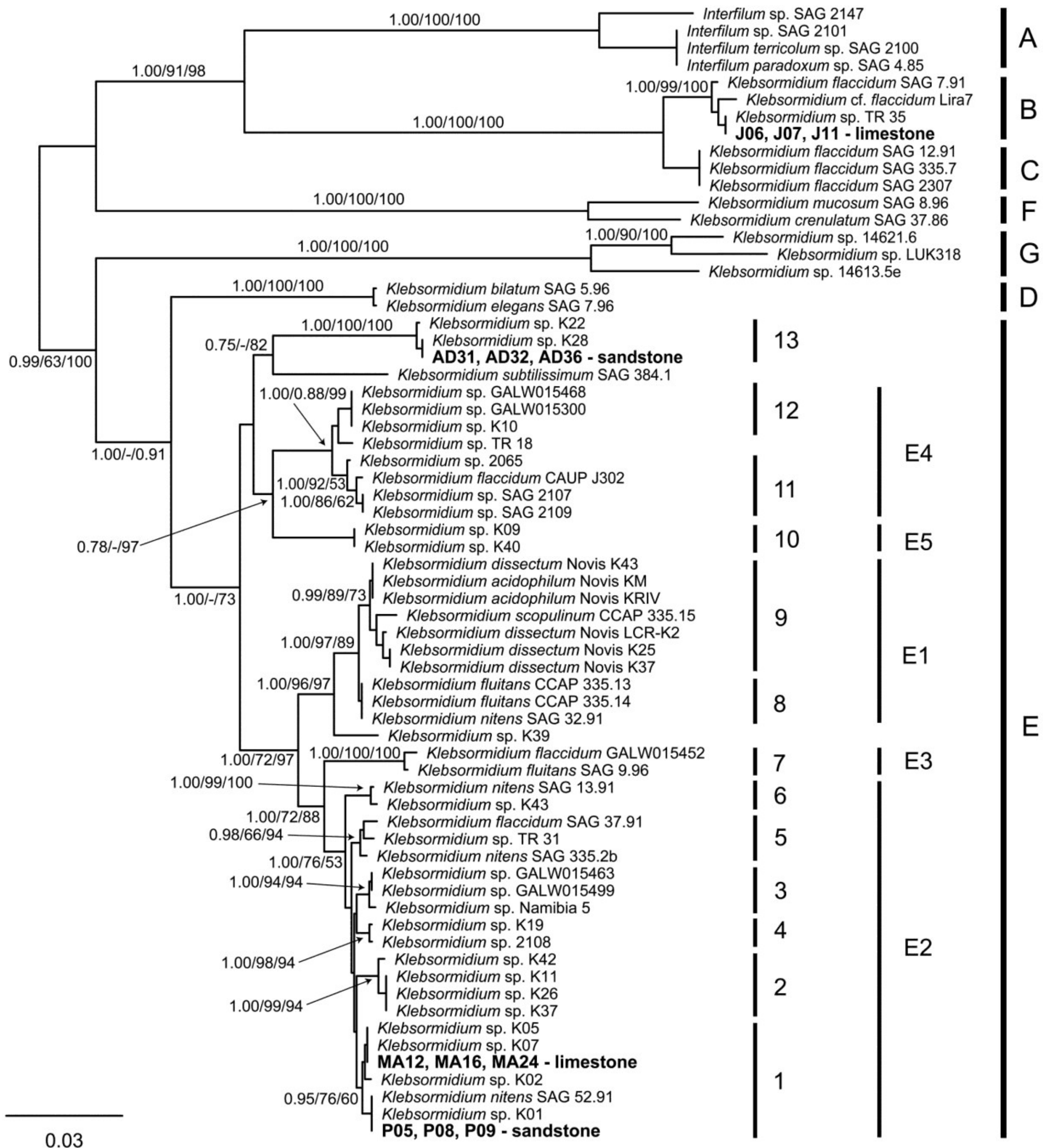
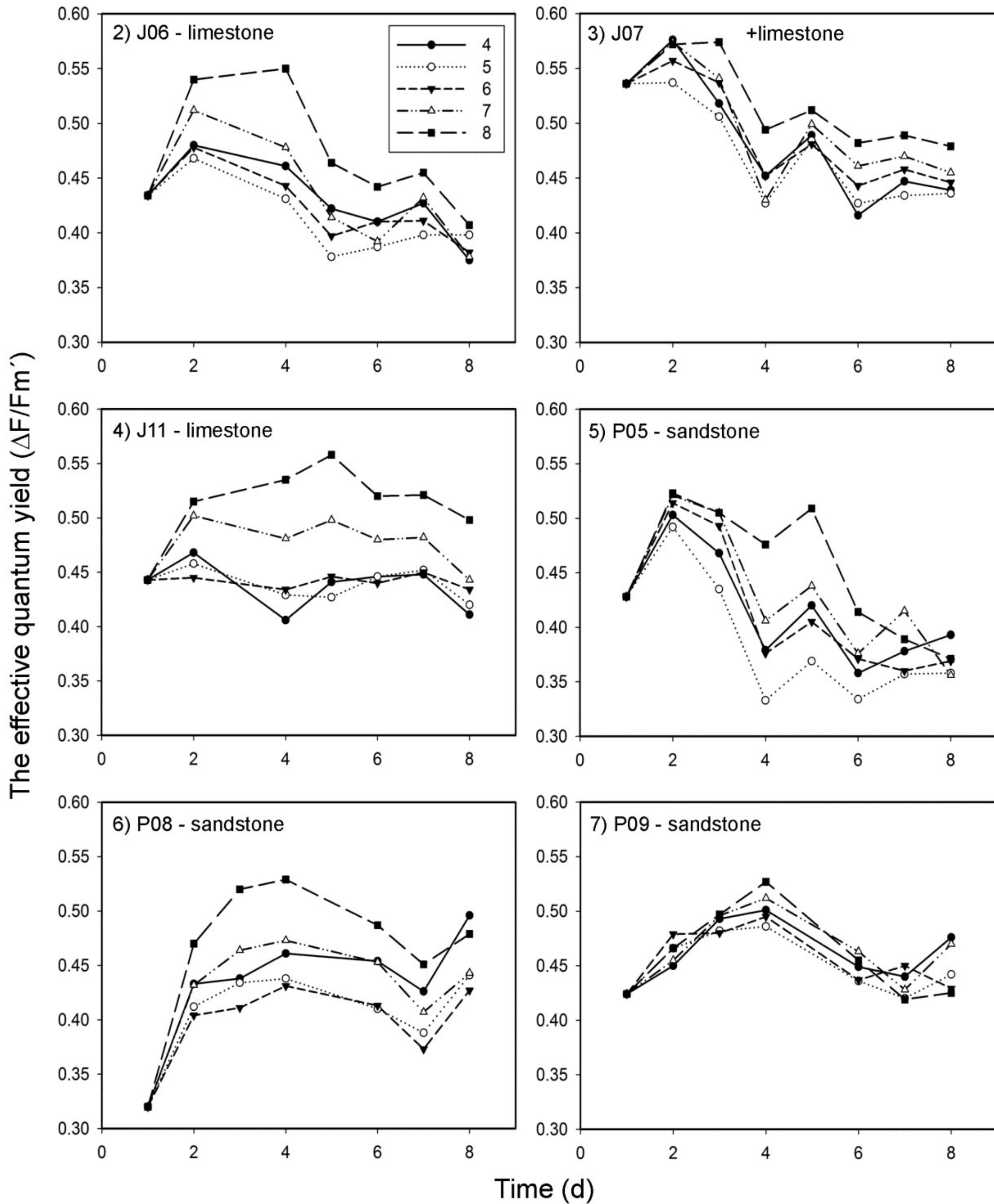
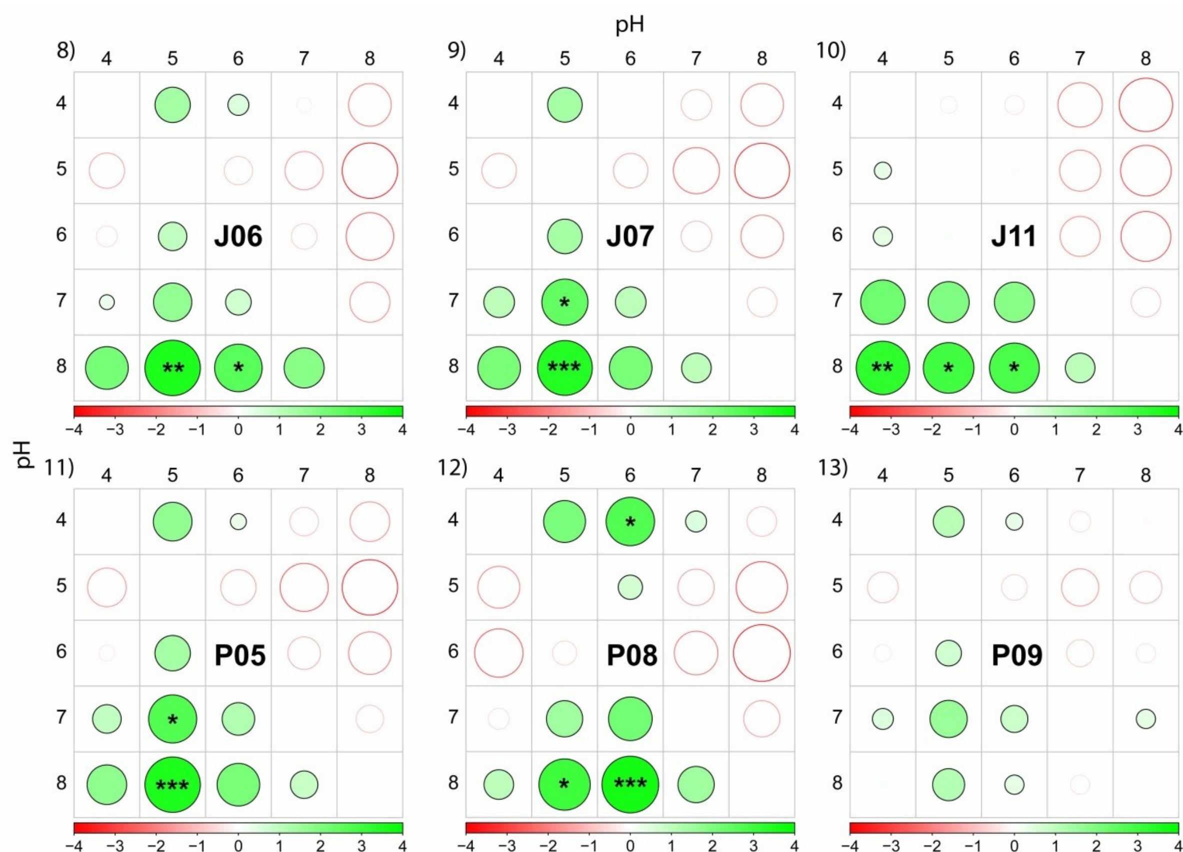


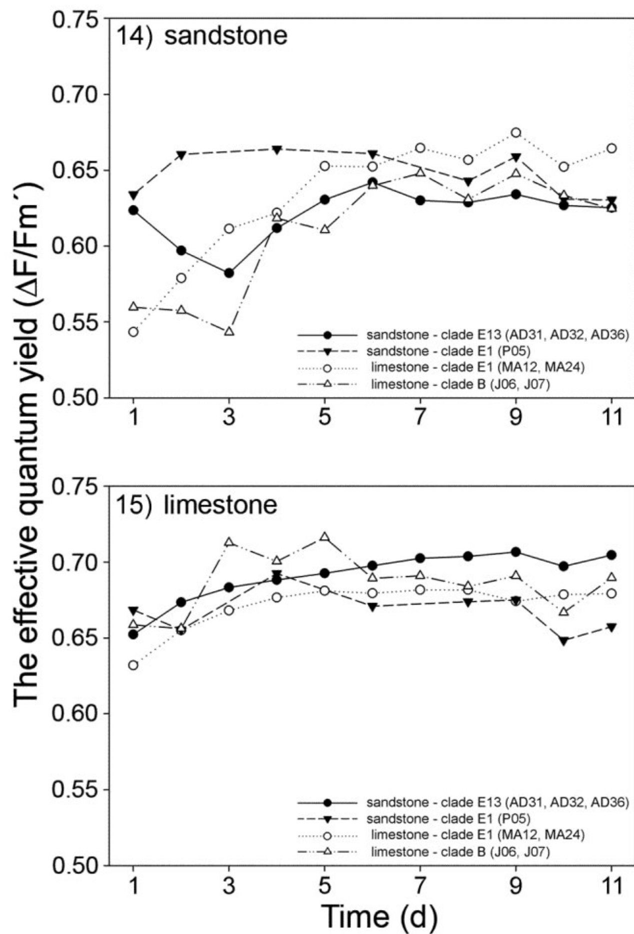
Figure 1. Phylogenetic tree obtained from Bayesian analysis based on *rbc*L dataset, showing the position of investigated strains of *Klebsormidium* and their relatives. Values at the nodes indicate statistical support estimated by MrBayes posterior probabilities (left), maximum likelihood bootstrap (middle), and maximum parsimony bootstrap (right). The clade numbering (A–G, E1–E6) follows Rindi et al. (2011) and clades (1–13) are according to Škaloud and Rindi (2013).



Figures 2–7. The effective quantum yield ($\Delta F/F_m'$) of PS II measured for three limestone (J06, J07, J11) and three sandstone (P05, P08, P09) strains of Klebsormidium at five different pH levels (4–8). The values plotted represent means of four replicated measurements. Differences were evaluated by post hoc comparisons of nonparametric Friedman two-way ANOVA tests (see Figs 8–13). Standard deviations of those measurements are given in Tables S1–S2.



Figures 8–13. Differences in the effective quantum yield ($\Delta F/F_m'$) of PS II measured at five different pH levels (4–8) on six selected strains (see Figs 2–7 for measured data). Differences were evaluated by post hoc comparisons of nonparametric Friedman two-way ANOVA tests. First, repeated $\Delta F/F_m'$ measurements at different pH values were ordered from highest to lowest. Then, mean ranks were calculated for every pH level. Within each row (a particular pH level), positive and negative differences in $\Delta F/F_m'$ mean ranks are displayed by a symbol size and shading. Filled and empty circles display positive and negative differences in mean ranks, respectively, varying from -4 to 4 (see the colour shade legend). For example, in the strain J06 mean rank in $\Delta F/F_m'$ at pH 4 was higher relative to pH 5 and 6 but lower relative to pH 8. Significant differences as determined by Friedman two-way ANOVA tests are indicated by asterisks (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).



Figures 14–15. The effective quantum yield ($\Delta F/F_m'$) of PS II measured for four limestone (MA12, MA24, J06, J07) and four sandstone (AD31, AD32, AD36, P05) strains when growing directly on sandstone (Fig. 14) and limestone (Fig. 15) rock substrate. The values plotted represent means of four replicated measurements. Genetically identical strains were displayed by a single value for better clarity. Differences were evaluated by post hoc comparisons of nonparametric Friedman two-way ANOVA tests (see Fig. 16). Standard deviations of the measurements are given in Tables S3–S4.

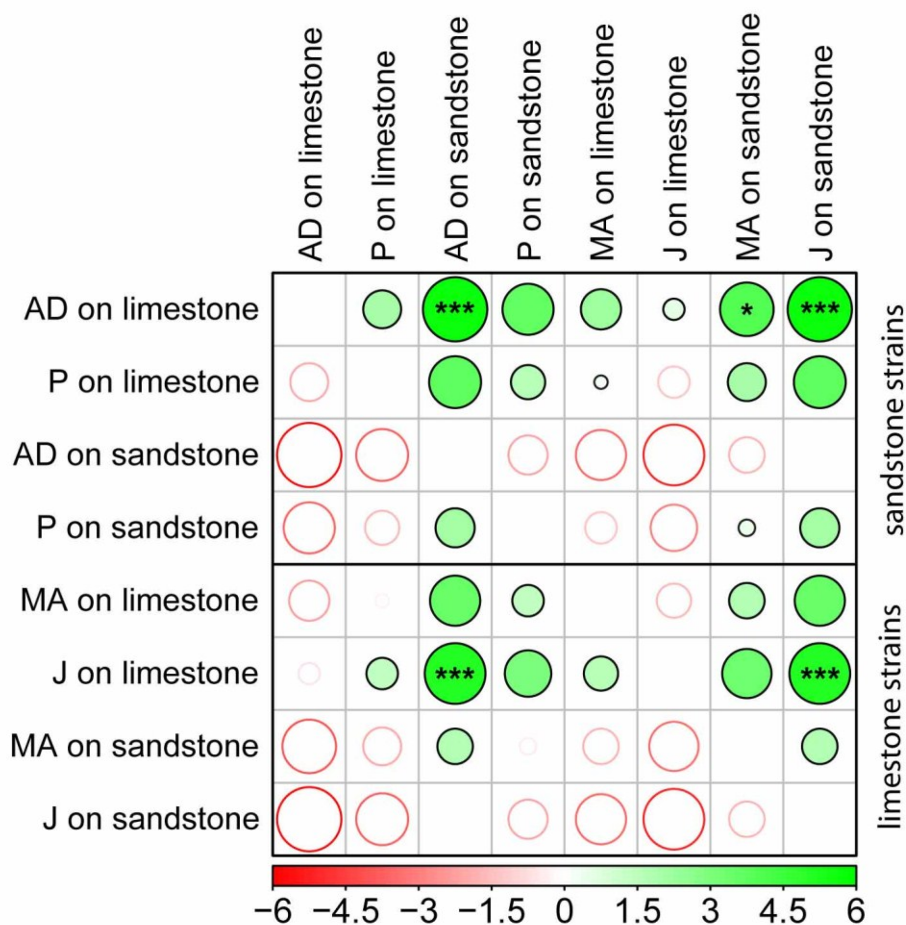
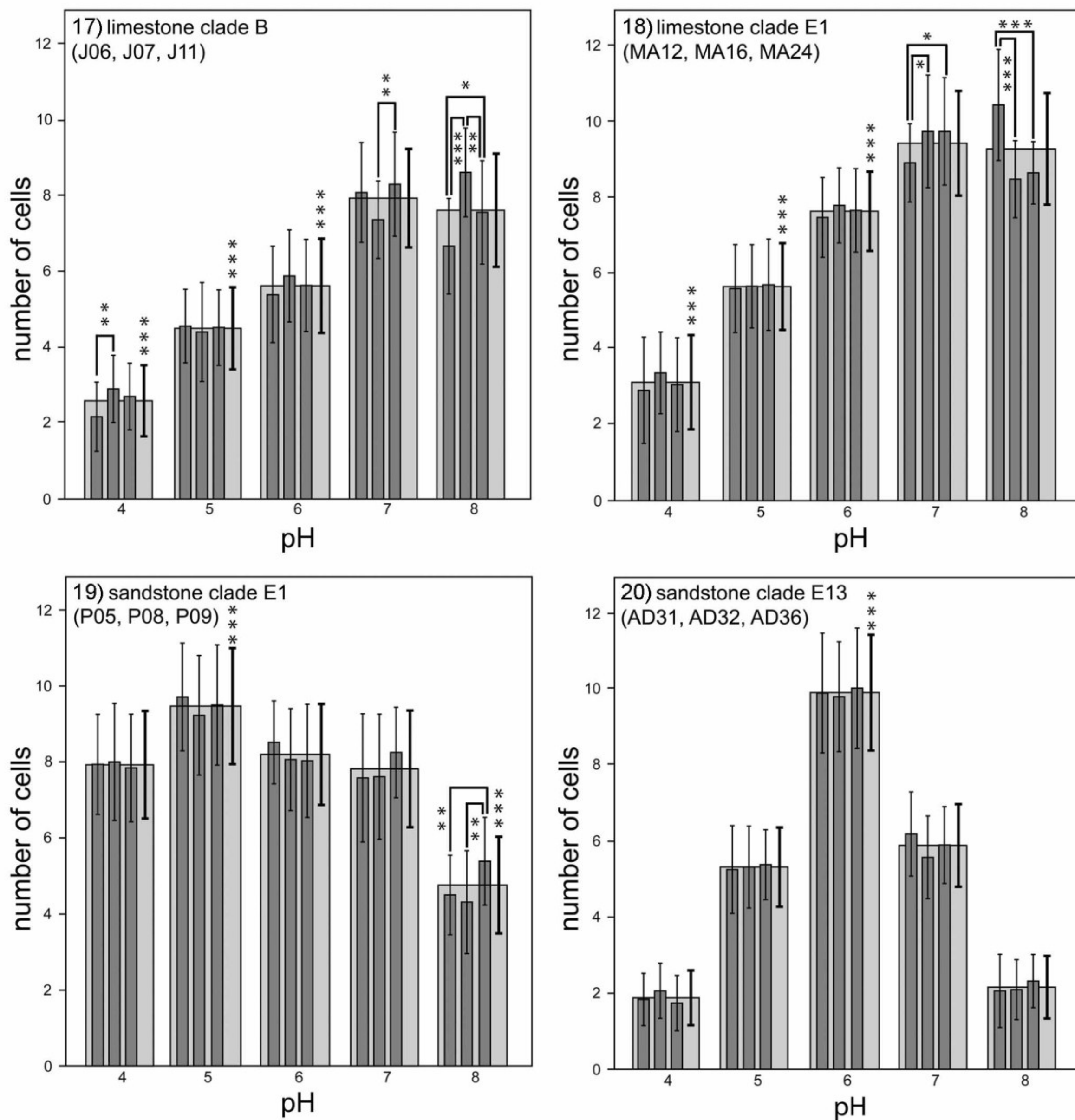


Figure 16. Differences in the effective quantum yield ($\Delta F/F_m'$) of PS II measured at two different natural substrate (sandstone and limestone) on eight selected strains (see Figs 14–15 for measured data). Differences were evaluated by post hoc comparisons of nonparametric Friedman two-way ANOVA tests (see Figs 8–13 legend for further explanation). Filled and empty circles display positive and negative differences in mean ranks, respectively, varying from -6 to 6 (see the colour shade legend). Significant differences as determined by Friedman two-way ANOVA tests are indicated by asterisks (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).



Figures 17–20. Growth response of four lineages of *Klebsormidium* (limestone clades B and E1; sandstone clades E1 and E13) to different pH levels (4–8). The graphs display the total number of cells in young filaments grown from single cells after 4 days of cultivation on agar plates. Each lineage is represented by three investigated strains. Dark grey bars display mean number of cells determined for each studied strain, light grey bars display the overall means for the lineage. The mean values were calculated from 30–40 replicates, and the standard deviations are displayed for each measurement. Significant differences as determined by one-way ANOVAs Tukey's pairwise comparisons are indicated by asterisks (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

SUPPLEMENTARY DATA

Table 1. Mean and standard deviations of the effective quantum yield of photosystem II in liquid medium for three limestone strains (J06, J07, J11).

Limestone strains										
J06	pH 4		pH 5		pH 6		pH7		pH 8	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.434	0.023	0.434	0.023	0.434	0.023	0.434	0.023	0.434	0.023
2	0.480	0.029	0.468	0.029	0.478	0.029	0.512	0.021	0.540	0.016
4	0.461	0.030	0.431	0.030	0.443	0.035	0.478	0.024	0.550	0.027
5	0.422	0.050	0.378	0.045	0.397	0.049	0.414	0.033	0.464	0.039
6	0.410	0.035	0.387	0.027	0.410	0.036	0.392	0.014	0.442	0.018
7	0.427	0.038	0.398	0.031	0.411	0.026	0.432	0.026	0.455	0.024
8	0.375	0.036	0.398	0.026	0.382	0.034	0.378	0.019	0.407	0.027
J07	pH 4		pH 5		pH 6		pH7		pH 8	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.536	0.014	0.536	0.014	0.536	0.014	0.536	0.014	0.536	0.014
2	0.576	0.019	0.537	0.018	0.557	0.016	0.574	0.018	0.572	0.022
3	0.518	0.025	0.506	0.014	0.537	0.013	0.541	0.017	0.575	0.014
4	0.452	0.028	0.427	0.022	0.452	0.023	0.430	0.026	0.494	0.027
5	0.489	0.017	0.485	0.015	0.481	0.033	0.499	0.010	0.512	0.017
6	0.416	0.025	0.427	0.023	0.443	0.019	0.461	0.017	0.482	0.026
7	0.447	0.028	0.434	0.034	0.458	0.029	0.470	0.017	0.489	0.022
8	0.439	0.020	0.436	0.016	0.446	0.020	0.455	0.019	0.479	0.026
J11	pH 4		pH 5		pH 6		pH7		pH 8	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.443	0.020	0.443	0.020	0.443	0.020	0.443	0.020	0.443	0.020
2	0.468	0.019	0.458	0.023	0.445	0.018	0.502	0.020	0.515	0.019
4	0.406	0.022	0.429	0.041	0.434	0.040	0.481	0.027	0.535	0.026
5	0.441	0.040	0.427	0.036	0.446	0.043	0.498	0.027	0.558	0.027
6	0.446	0.018	0.446	0.029	0.440	0.019	0.480	0.016	0.520	0.025
7	0.448	0.019	0.452	0.023	0.450	0.027	0.482	0.015	0.521	0.024
8	0.411	0.037	0.420	0.028	0.434	0.023	0.443	0.036	0.498	0.021

Table 2. Mean and standard deviations of the effective quantum yield of photosystem II in liquid medium for three sandstone strains (P05. P08. P09).

Sandstone strains										
P05	pH 4		pH 5		pH 6		pH7		pH 8	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.428	0.024	0.428	0.024	0.428	0.024	0.428	0.024	0.428	0.024
2	0.503	0.010	0.492	0.022	0.514	0.024	0.521	0.023	0.523	0.024
3	0.468	0.018	0.435	0.024	0.493	0.010	0.505	0.015	0.505	0.030
4	0.379	0.020	0.333	0.018	0.376	0.023	0.406	0.019	0.476	0.021
5	0.420	0.024	0.369	0.021	0.405	0.023	0.438	0.029	0.507	0.024
6	0.358	0.030	0.334	0.021	0.371	0.029	0.376	0.019	0.414	0.035
7	0.378	0.014	0.357	0.031	0.360	0.031	0.415	0.034	0.389	0.061
8	0.393	0.047	0.358	0.025	0.369	0.022	0.356	0.012	0.371	0.018
P08	pH 4		pH 5		pH 6		pH7		pH 8	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.320	0.042	0.320	0.042	0.320	0.042	0.320	0.042	0.320	0.042
2	0.433	0.024	0.412	0.016	0.404	0.024	0.432	0.023	0.470	0.010
3	0.438	0.012	0.434	0.027	0.411	0.022	0.464	0.013	0.520	0.024
4	0.461	0.013	0.438	0.016	0.431	0.028	0.473	0.016	0.529	0.021
6	0.454	0.036	0.410	0.031	0.413	0.035	0.453	0.039	0.487	0.036
7	0.426	0.038	0.388	0.027	0.373	0.019	0.407	0.035	0.451	0.036
8	0.496	0.023	0.441	0.029	0.427	0.032	0.443	0.018	0.479	0.043
P09	pH 4		pH 5		pH 6		pH7		pH 8	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.424	0.022	0.424	0.022	0.424	0.022	0.424	0.022	0.424	0.022
2	0.450	0.023	0.466	0.020	0.479	0.023	0.455	0.021	0.466	0.026
3	0.493	0.081	0.482	0.029	0.480	0.022	0.496	0.014	0.497	0.031
4	0.501	0.023	0.486	0.019	0.495	0.013	0.512	0.016	0.527	0.017
6	0.449	0.030	0.436	0.033	0.437	0.046	0.463	0.034	0.455	0.039
7	0.440	0.020	0.421	0.022	0.450	0.018	0.428	0.019	0.419	0.014
8	0.476	0.015	0.442	0.047	0.429	0.018	0.470	0.019	0.425	0.040

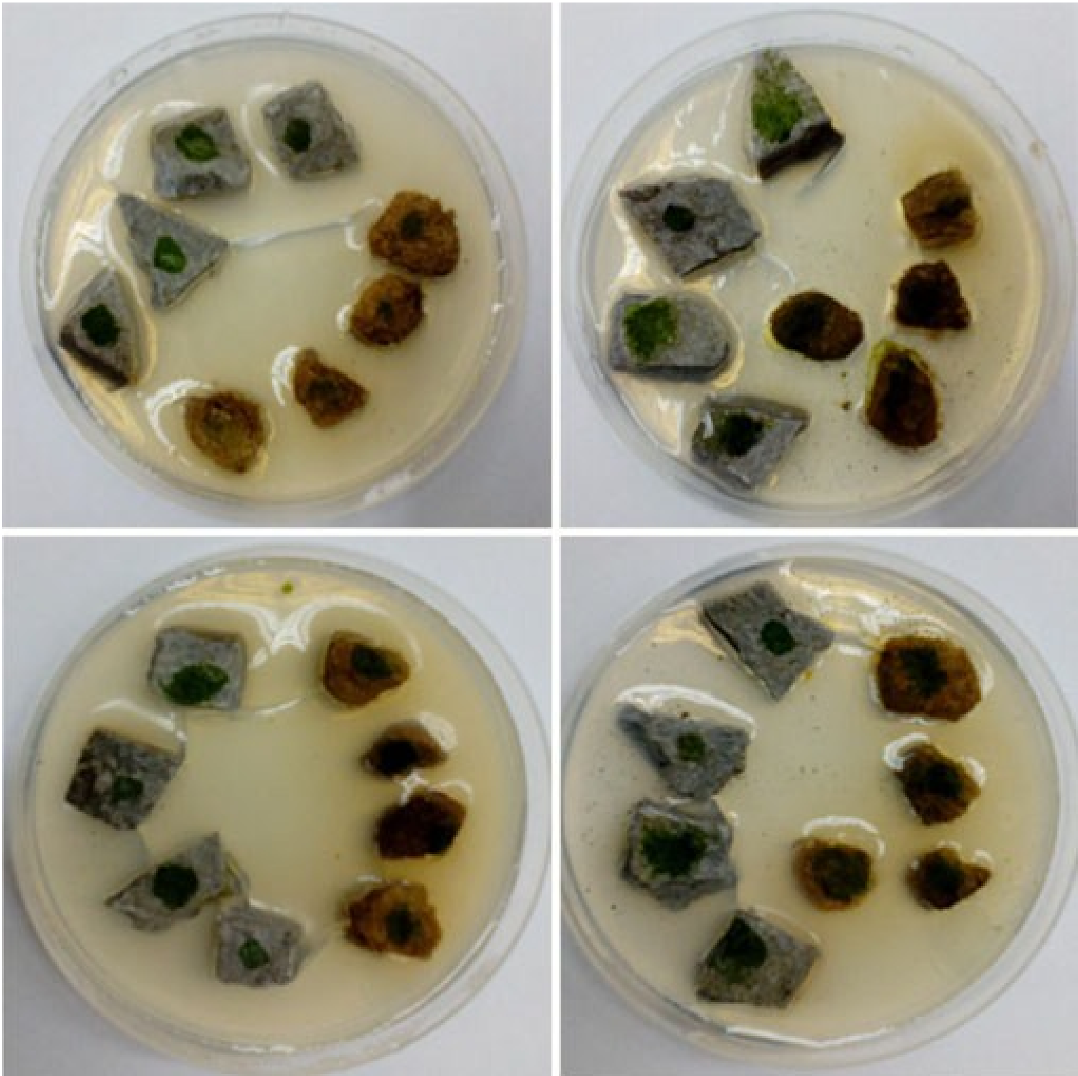
Table 3. Mean and standard deviations of the effective quantum yield of photosystem II for strains growth on sandstone substrate.

	Sandstone strains											
	AD31		AD32		AD36		AD		P05		P	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.544	0.038	0.659	0.059	0.639	0.024	0.614	0.040	0.635	0.008	0.635	0.008
2	0.433	0.210	0.680	0.036	0.615	0.024	0.576	0.090	0.661	0.008	0.661	0.008
3	0.424	0.284	0.644	0.110	0.666	0.010	0.578	0.135	-	-	-	-
4	0.464	0.266	0.680	0.038	0.685	0.006	0.609	0.103	0.666	0.013	0.666	0.013
5	0.503	0.212	0.697	0.030	0.698	0.009	0.633	0.084	-	-	-	-
6	0.525	0.243	0.701	0.020	0.687	0.020	0.638	0.094	0.662	0.003	0.662	0.003
7	0.491	0.328	0.702	0.018	0.705	0.010	0.633	0.118	-	-	-	-
8	0.481	0.321	0.713	0.026	0.702	0.007	0.632	0.118	0.643	0.008	0.643	0.008
9	0.492	0.329	0.711	0.032	0.709	0.014	0.637	0.125	0.658	0.006	0.658	0.006
10	0.504	0.336	0.707	0.032	0.686	0.012	0.632	0.127	0.636	0.016	0.636	0.016
11	0.485	0.325	0.700	0.030	0.701	0.008	0.629	0.121	0.632	0.008	0.632	0.008
	Limestone strains											
	MA12		MA24		MA		J06		J07		J	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.574	0.096	0.513	0.098	0.543	0.097	0.503	0.072	0.616	0.025	0.560	0.048
2	0.552	0.168	0.606	0.045	0.579	0.107	0.479	0.074	0.640	0.014	0.560	0.044
3	0.602	0.117	0.621	0.043	0.611	0.080	0.543	0.075	-	-	0.543	0.075
4	0.618	0.058	0.626	0.026	0.622	0.042	0.574	0.085	0.659	0.014	0.616	0.049
5	0.638	0.037	0.668	0.015	0.653	0.026	0.611	0.088	-	-	0.611	0.088
6	0.651	0.026	0.654	0.020	0.652	0.023	0.622	0.062	0.653	0.019	0.637	0.040
7	0.675	0.024	0.654	0.016	0.665	0.020	0.648	0.054	-	-	0.648	0.054
8	0.662	0.011	0.652	0.022	0.657	0.016	0.617	0.092	0.643	0.014	0.630	0.053
9	0.676	0.008	0.673	0.021	0.675	0.014	0.647	0.052	0.642	0.016	0.645	0.034
10	0.658	0.025	0.647	0.012	0.652	0.019	0.646	0.027	0.622	0.020	0.634	0.023
11	0.664	0.006	0.665	0.010	0.664	0.008	0.633	0.051	0.626	0.016	0.629	0.034

Table 4. Mean and standard deviations of the effective quantum yield of photosystem II for strains growth on limestone substrate.

	Sandstone strains											
	AD31		AD32		AD36		AD		P05		P	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.622	0.038	0.668	0.071	0.668	0.032	0.652	0.047	0.664	0.018	0.664	0.018
2	0.669	0.023	0.674	0.060	0.678	0.006	0.674	0.030	0.661	0.017	0.661	0.017
3	0.682	0.029	0.690	0.035	0.678	0.010	0.683	0.025	-	-	-	-
4	0.684	0.013	0.689	0.039	0.692	0.011	0.688	0.021	0.693	0.014	0.693	0.014
5	0.679	0.013	0.706	0.029	0.692	0.012	0.693	0.018	-	-	-	-
6	0.670	0.038	0.722	0.015	0.700	0.019	0.698	0.024	0.667	0.027	0.667	0.027
7	0.699	0.040	0.711	0.008	0.698	0.008	0.703	0.019	-	-	-	-
8	0.696	0.027	0.723	0.011	0.693	0.015	0.704	0.018	0.673	0.011	0.673	0.011
9	0.692	0.044	0.728	0.001	0.700	0.008	0.707	0.018	0.677	0.010	0.677	0.010
10	0.695	0.022	0.727	0.005	0.670	0.028	0.697	0.018	0.648	0.013	0.648	0.013
11	0.690	0.030	0.733	0.008	0.691	0.009	0.705	0.016	0.654	0.015	0.654	0.015
	Limestone strains											
	MA12		MA24		MA		J06		J07		J	
day	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations	mean	standard deviations
1	0.652	0.015	0.612	0.011	0.632	0.013	0.648	0.014	0.672	0.018	0.660	0.016
2	0.673	0.007	0.638	0.014	0.655	0.010	0.642	0.026	0.673	0.010	0.657	0.018
3	0.669	0.022	0.668	0.005	0.668	0.014	0.713	0.020	-	-	0.713	0.020
4	0.679	0.008	0.675	0.004	0.677	0.006	0.708	0.012	0.696	0.011	0.702	0.011
5	0.690	0.012	0.672	0.003	0.681	0.008	0.716	0.012	-	-	0.716	0.012
6	0.674	0.019	0.686	0.013	0.680	0.016	0.701	0.040	0.672	0.017	0.686	0.028
7	0.682	0.007	0.681	0.010	0.682	0.008	0.691	0.044	-	-	0.691	0.044
8	0.686	0.006	0.678	0.011	0.682	0.009	0.683	0.062	0.679	0.014	0.681	0.038
9	0.670	0.013	0.678	0.009	0.674	0.011	0.699	0.039	0.682	0.012	0.691	0.025
10	0.667	0.012	0.690	0.009	0.679	0.011	0.678	0.072	0.658	0.016	0.668	0.044
11	0.667	0.019	0.692	0.007	0.679	0.013	0.689	0.044	0.687	0.011	0.688	0.028

Figure 1. Set up an experiment to measure the effective quantum yield of photosystem II for strains growth on natural substrate.



3.5.Paper 5

Rindi F., **Ryšánek D**. and Škaloud P.

Problems of epitypification in morphologically simple green microalgae: a case study of two widespread species of *Klebsormidium* (Klebsormidiophyceae, Streptophyta).

Fottea (submitted)

Problems of epitypification in morphologically simple green microalgae: a case study of two widespread species of *Klebsormidium* (Klebsormidiophyceae, Streptophyta).

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ABSTRACT

The extensive genetic cryptic diversity revealed by molecular studies in many green microalgae has caused great uncertainties in the circumscription of some species described on strictly morphological basis. These uncertainties should be resolved obtaining molecular data from type materials, but this procedure is not feasible in many species because the type specimens are illustrations, or too small or poorly preserved to obtain DNA sequence data. In these situations, the selection of an epitype is often a mandatory requirement to define the identity of a species and establish unambiguously its position in molecular phylogenies. In this study we investigated the identity of two widespread species of *Klebsormidium* (Streptophyta), *K. flaccidum* and *K. nitens*, which were recently epitypified by Mikhailyuk and colleagues. We collected several specimens of these algae from the type localities and the original habitats of these two species, for which we examined morphology in the field material and in culture, and obtained sequences of the *rbc*L gene. On the basis of the original descriptions, we conclude that the designation of the epitype of *K. flaccidum* was correct, whereas the epitype of *K. nitens* (which consists of material collected tens of thousands of km from the type locality) was most probably incorrect. We discuss the implications of these decisions for the classification of *Klebsormidium* and, more generally, the importance of the correct choice of epitype material for the taxonomy of green microalgae.

INTRODUCTION

In recent decades, the introduction of DNA sequence data in studies of systematics and taxonomy has revolutionized the classification of all groups of algae. The impact of these methods has been particularly strong on algal groups with simple morphologies, resulting from an evolutionary history characterized by high levels of morphological homoplasy. The use of molecular data has greatly advanced our understanding of the genetic diversity of these organisms and has been of substantial help for species circumscription, particularly in taxa where morphology is strongly affected by phenotypic plasticity related to environmental conditions. In numerous cases, however, the contrasting scenarios drawn by morphological and molecular data have been source of major nomenclatural problems, that for some taxa have not yet been resolved.

Green microalgae living in freshwater and terrestrial habitats (belonging to the Chlorophyta and Streptophyta lineages; LELIAERT et al. 2012) are perhaps the most suitable group to exemplify these difficulties. These organisms have a simple morphology that is preferable to three main different habits: single cells; uniseriate filaments, either branched or not; few-celled colonies, of various shape (RINDI 2011). Based on molecular data produced in the last 20 years, it is now clear that rampant morphological convergence is a typical feature in the evolution of these algae, producing almost identical morphologies in unrelated species (HUSS et al. 1999; KRIENITZ & BOCK 2012; FUČÍKOVÁ et al. 2014; KRIENITZ et al. 2015). Several genera, apparently well-defined from a morphological point of view, were recovered as polyphyletic or paraphyletic in molecular phylogenies (e.g., *Chlorella* BEIJ. - HUSS et al. 1999; *Dictyosphaerium* NÄGELI - BOCK et al. 2011; *Pediastrum* MEYEN - BUCHHEIM et al. 2005; *Printzina* R.H.THOMPSON et D.E.WUJEK and *Trentepohlia* MART. - RINDI et al. 2009; *Trebouxia* PUYM. - ŠKALOUD & PEKSA 2010). In the recent past this called for major taxonomic reassessments that resulted in the description of numerous new taxa; in particular, the splitting of a genus into multiple genera, or a species into multiple species, has been a widespread situation (e.g., HUSS et al. 1999; BOCK et al. 2011; ALLEWAERT et al. 2015; ŠKALOUD et al. 2015). A common problem arising in these cases is to link correctly Linnean names with clades recovered in molecular phylogenies (VERBRUGGEN 2014), which is a fundamental requirement in order to apply correctly names of species and genera. The only definitive way to establish such a link is to obtain sequences from the type specimen or strain (the only one to which a name is unambiguously associated), and establish its placement in molecular phylogenies. In general, this is not a problem for green microalgae described in the last 10-15 years, for which the description is usually based on both morphological and

molecular data, and sequences from type strains are available. However, many common green microalgae were described by early phycologists (e.g., LINNAEUS 1753, 1759; AGARDH 1824; KÜTZING 1843, 1849; NÄGELI 1849) based only on morphological characters. For these, apart for a few lucky exceptions in which the original culture is still available (e.g., *Chlorella vulgaris* BEIJ., the type species of the genus *Chlorella*; KRIENITZ et al. 2015) or the type is a relatively large and well conserved specimen (e.g., *Prasiola crispa* (LIGHTFOOT) KÜTZING, MONIZ et al. 2012), the type specimen/strain cannot be used for DNA extraction and sequencing, usually because the material is either too small or is an illustration. Problems of this type have the potential to lead to a situation of taxonomic paralysis and can be resolved only with the designation of a carefully selected epitype, whose morphology agrees in full with the original protologue and the type specimen.

The genus *Klebsormidium* P.C. SILVA, MATTOX et W.H. BLACKWELL includes 21 species of green algae distributed in a wide range of terrestrial and freshwater habitats (ETTL & GÄRTNER 1995; LOKHORST 1996; ŠKALOUD 2006), classified in the family Klebsormidiaceae, order Klebsormidiales (Streptophyta). Morphologically, *Klebsormidium* is characterized by uniseriate unbranched filaments formed by cells containing a single parietal chloroplast with a pyrenoid, reproducing only by biflagellate zoospores produced in undifferentiated cells and released through an ostiole in the cell wall (ETTL & GÄRTNER 1995; LOKHORST 1996; GUIRY & GUIRY 2015). These features make members of this genus readily recognizable. However, it has long been acknowledged that identification at species level is complicated by the scarcity of useful morphological characters and the phenotypic plasticity that affects some of them. In the last 10 years, molecular data on *Klebsormidium* have been produced in plentiful amounts (NOVIS 2006, MIKHAILYUK et al. 2008; RINDI et al. 2008, 2011; KAPLAN et al. 2012; KARSTEN et al. 2013; ŠKALOUD & RINDI 2013; ŠKALOUD et al. 2014; MIKHAILYUK et al. 2015; RYŠÁNEK et al. 2015, 2016a, 2016b), revealing a great deal of genetic diversity and the paraphyly of the genus as defined on morphological basis, due to the inclusion in it of *Interfilum* R. CHODAT, a genus that shares with *Klebsormidium* many ultrastructural and developmental features (MIKHAILYUK et al. 2014) but consists of packet-like mucilaginous colonies (MIKHAILYUK et al. 2008, 2014). This has greatly confused the circumscription of some species, including the type species *Klebsormidium flaccidum* (KÜTZ.) P.C. SILVA, MATTOX et W.H. BLACKWELL. The original description of this alga (as *Ulothrix flaccida* KÜTZ.; KÜTZING 1849) is brief and does not provide important morphological details. So, the morphological circumscription of the species has been largely shaped by descriptions provided by subsequent authors (RABENHORST 1868; HANSGIRG 1886; DE TONI 1889; HAZEN

1902; MATTOX & BOLD 1962; PRINTZ 1964; RAMANATHAN 1964; ETTL & GÄRTNER 1995; LOKHORST 1996). Sometimes descriptions provided by different authors disagree with regard to some morphological characters, and only very few authors (*e.g.*, LOKHORST 1996) appear to have examined the type material; seemingly, none of them tried to recollect this alga from the original type locality and habitat (stony streets in Strasbourg, France). In molecular phylogenies, strains identified as *K. flaccidum* are scattered in many different clades, making this species polyphyletic (RINDI et al. 2008, 2011; ŠKALOUD & RINDI 2013; ŠKALOUD et al. 2014; RYŠÁNEK et al. 2015). The same situation applies to another common species of *Klebsormidium*, *K. nitens* (MENEGH. IN KÜTZ.) LOKHORST. An additional complication in this case is represented by the fact that the type locality was not designated. The original description (MENEGHINI IN KÜTZING 1849: 349) does not make any mention of the original collection site and the specimen designated by LOKHORST (1996) as type does not provide any information that may be of help in this regard.

These uncertainties required a reassessment of the identity of both *K. flaccidum* and *K. nitens* based on the designation of epitypes for which molecular data are available. In a recent study, MIKHAILYUK et al. (2015) resolved this problem proposing epitypes for several *Klebsormidium* species, including *K. flaccidum and K. nitens*. To epitypify the two species, these authors selected strains isolated and investigated by LOKHORST (1996), who provided the most recent monographic treatment of the genus. However, the work of LOKHORST (1996) was published at a time when molecular data for *Klebsormidium* were not available and none of the strains selected were isolated from type localities or presumptive collection areas (i.e., France and Italy). In fact, the strains SAG 2307 (epitype of *K. flaccidum*) and SAG 13.91 (epitype of *K. nitens*) were obtained from samples collected respectively in Niederkruechten, Germany, and Tekoa, New Zealand.

Recently, we had the possibility to make collections of terrestrial algae in the type localities of *K. flaccidum* and *K. nitens*. We collected there samples of *Klebsormidium* from habitats corresponding to the original descriptions and we selected among them strains in morphological agreement with the original protologues. In this study we characterize morphologically and genetically these strains, comparing them with the to the epitypes proposed by MIKHAILYUK et al. (2015). Based on this comparison, we discuss some general implications for epitypification of morphologically simple green microalgae.

MATERIALS AND METHODS

Collections and cultivation.

A strain of *K. flaccidum* was collected by PŠ on 28 May 2014 in Strasbourg, France, the type locality of *Ulothrix flaccida*. A mixed sample was collected by scraping several green crusts from an old stone block pavement, in la Place du Marché Gayot (48.5828 N 7.7531 E). The samples were placed in plastic sampling vials, and transferred to the laboratory. Search for authentic *Klebsormidium nitens* was made by FR on 15 November 2014 in the botanical garden of Padua, Italy (45.399 N 11.880 E), a site that can be considered presumptive type locality of *Ulothrix nitens* MENEGH. IN KÜTZ. (see Discussion). Samples were collected by scraping green crusts from flowerpots (the type of habitat mentioned in the original description), margins of pools and old walls. The samples were placed in plastic ziploc bags and mailed to PŠ in sealed envelopes. In the laboratory, the samples were spread on Petri dishes with agarized BBM medium (BISCHOFF & BOLD 1963), and cultivated at 25°C under a constant photon irradiance of 34 μmol m^{-2} s^{-1} provided by cool fluorescent tubes (Philips Master TL5 HE 21W/840, Royal Philips Electronics, Amsterdam, the Netherlands). Algal microcolonies grown up after 5-10 weeks were transferred repeatedly to fresh Petri dishes. After three changes of each isolate to fresh Petri dishes, the obtained cultures were observed to be unialgal by examination under a light microscope. Unialgal stock cultures of *Klebsormidium* were maintained in liquid BBM, under the conditions described above.

DNA extraction, sequencing, and phylogenetic analyses.

DNA was isolated according to the protocol published by RYŠÁNEK et al. (2015), and stored at −20°C. Sequences of the *rbc*L gene, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, were obtained by polymerase chain reaction (PCR) amplification with a Touchgene Gradient cycler (Techne, United Kingdom). The *rbc*L gene was amplified by using the newly designed primer KF590 (5′-GAT GAA AAC GTA AAC TCT CAG C-3′) and the primer rbcL-KR2 (5′-GGT TGC CTT CGC GAG CTA-3′; ŠKALOUD & RINDI 2013). Each 20-μL reaction for PCR was conducted as described by RYŠÁNEK et al. (2015). The PCR protocol followed ŠKALOUD & RINDI (2013). Sequencing reads were assembled and edited using the SeqAssem software (HEPPERLE 2004).

For phylogenetic analyses, the newly obtained *rbc*L sequences were incorporated into previously aligned matrices (ŠKALOUD et al. 2014), and aligned by using ClustalW (THOMPSON et al. 1994) with MEGA version 6.0 (TAMURA et al. 2011). The *rbc*L alignment had 596 bp and is available on http://botany.natur.cuni.cz/algo/align/04_Klebsormidium.fas.

The aligned data set was analyzed by using Maximum Parsimony (MP) analysis with PAUP 4.0b10 (SWOFFORD 2002), Maximum Likelihood analysis (ML) with GARLI (ZWICKL 2006), and Bayesian analysis (BI) with MrBayes version 3.2.2 (RONQUIST et al. 2012). The evolutionary model applied in ML and BI analyses was determined by using PAUP/MrModeltest 2.3 (NYLANDER 2004). The model selected under the Akaike Information Criterion was GTR + I + G. The BI analysis was performed by using the priors set as default in MrBayes. The robustness of the tree topologies was assessed by bootstrapping the data set as described by ŠKALOUD & RINDI (2013).

Morphological observations.

To obtain a detailed morphological characterization of *Klebsormidium* isolates, the strains were regularly observed during the 3-month period of culturing, using an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) equipped with a differential interference contrast. In addition to observing the general growth habit, formation of H-shaped pieces at cross walls, and reproductive features (shape of release aperture in lateral wall of zoosporangial cell and germination pattern of zoospores), cell dimensions were measured in detail in ImageJ software. For each strain, cell dimensions were measured on fresh, 4-week-old, exponentially growing cultures, as well as on nutrient-depleted, 3-month-old cultures. The average, minimal and maximal values were calculated from at least 30 replicates. Zoospore formation was induced by transferring the cultures to a 1% glucose solution, and keeping them in darkness at a temperature of about 10°C.

RESULTS

In total, we isolated 5 and 18 unialgal *Klebsormidium* strains from the material collected in Strasbourg, France and Padua, Italy, respectively. The five Strasbourg strains were labelled as S201, S202, S203, S204, and S205. The Padua strains were isolated from material collected in six sampling sites: side of Basilica of St. Antonio (strains F1A, F1B, and F1C), and five sites in the Botanical Garden: crust on stony manufacture (strains F2A and F2B), concrete manufacture (strains F3A, F3B, F3C, F3D, and F3E), concrete margin of a pond (F7A), concrete side of a pond (F8A, F8B, and F8C), and base of a statue (F11A, F11B, F11C, and F11D). All isolated strains were subjected to subsequent molecular analyses.

Molecular data.

Bayesian analysis of the *rbc*L dataset (Fig. 1) resulted in the topology largely corresponding to the analyses published in several previous works (RINDI et al. 2011; ŠKALOUD & RINDI 2013; ŠKALOUD et al. 2014). Our analysis resolved all superclades (A-G) and lineages (1-13) identified by RINDI et al. (2011) and ŠKALOUD & RINDI (2013), respectively. All strains isolated from the sample collected in Strasbourg, France (S201, S202, S203, S204, and S205) were inferred within a single lineage, the superclade C *sensu* RINDI et al. (2011). The strains from Padua, Italy were inferred within three different clades. The vast majority of them (15 strains labelled F1A-C, F2A-B, F3A-E, F8B, F11A-D) were inferred as members of the clade E4 *sensu* RINDI et al. (2011), i.e. clade 12 *sensu* ŠKALOUD & RINDI (2013). Strains F7A and F8A were inferred to belong to clade E2 *sensu* RINDI et al. (2011), i.e. clade 3 sensu ŠKALOUD & RINDI (2013). Finally, the strain F8C was resolved as a member of clade E3 *sensu* RINDI et al. (2011), i.e. clade 7 *sensu* Škaloud & Rindi (2013).

Morphological data.

For the purpose of detailed morphological characterization of the dominant lineages found in Strasbourg and Padua, three *Klebsormidium* cultures were selected from a set of cultured strains belonging to clades C and E4, respectively. Strasbourg *Klebsormidium* was represented by strains S201, S202, and S203. Filaments were very long, with more than 1,000 cells per filament, without any tendency to fragmentation in older cultures. Cells were cylindrical, not constricted, 0.7-2.6 times as long as wide (Figs 2A-C). Cell dimensions differed among the strains (Table 1): cells of the strain S201 were generally wider (7.4 ± 0.7 μm) and shorter (9.6 ± 3.6 μm), whereas the strain S202 produced generally thinner (6.3 ± 0.7 μm) and longer (12.6 ± 3.5 μm) cells. In all strains, cell width gradually increased with the age of the cultures. The most distinctive difference was observed in the strain S201: the average cell width increased from 6.8 ± 0.2 μm to 8.0 ± 0.6 μm after two months in culture. In mature cultures, formation of H-shaped pieces at cross walls was commonly observed in all strains (Fig. 2D). The release of zoospores was successfully induced in all observed strains. Whereas the release apertures in empty zoosporangial cell walls were distinct in the strain S202 (Fig. 2E), the remaining ones exhibited an indistinct margin of the apertures (Fig. 2F). In all strains, the zoospores predominantly germinated by bipolar pattern (Fig. 2G).

For morphological characterization of Padua strains, we selected the isolates F1B, F3C and F8B, inferred within the predominant clade E4. The strains generally shared a common

general morphology. Filaments were very long, with more than 1,000 cells per filament, without any tendency to fragmentation in older cultures (Figs 2H-J). Individual filaments were occasionally coiled together to form rope-like structures (Fig. 2J). Cells were cylindrical, not constricted, 0.8-1.9 times as long as wide (Figs 2H-I). Cell dimensions were comparable among the strains, with the exception of slightly wider cells in strain F8B (Table 1). Contrarily to the Strasbourg isolates, the cell width gradually decreased in aging cultures. In all strains, the cell width decreased by 0.6-0.9 μm after two months of cultivation. Similarly, the average cell length increased by 2-3 μm. The formation of H-shaped cell wall pieces was never observed. In all cultures, we observed the occasional formation of spirally twisted filaments resulting in the production of rope-like strands of filaments (Fig. 2J). Despite several attempts, production of zoospores could not be induced.

DISCUSSION

In the present study, we made collections of *Klebsormidium* from the type locality of *Klebsormidium flaccidum* (Strasbourg) and a locality that is the best candidate as putative type locality for *K. nitens* (Padua). Based on our observations and the original descriptions of the two species, we isolated some strains that we believe to correspond to the genuine *Ulothrix flaccida* and *Ulothrix nitens* of Kützing (KÜTZING 1849). It is worthy to stress that *K. flaccidum* and *K. nitens* are species of particular significance, and their correct taxonomic circumscription is critically important from several points of view. The phylogenetic position of these algae affects the classification of the whole order Klebsormidiales, a group that occupies a basal position in the streptophycean lineage (LEWIS & MCCOURT 2004; LELIAERT et al. 2012) and is considered of great evolutionary and genomic interest (CIVÁŇ et al. 2014; HORI et al. 2014). Especially important in this regard is the identity of *K. flaccidum*, the type species of *Klebsormidium*. In molecular phylogenies, several strains corresponding morphologically to *K. flaccidum* occur in the clade that is sister to *Interfilum* (MIKHAILYUK et al. 2008; RINDI et al. 2008, 2011; ŠKALOUD et al. 2014; RYŠÁNEK et al. 2015), whereas many other strains occur in separate clades. Since *Klebsormidium* is made paraphyletic by *Interfilum*, and *K. flaccidum* is the type species of *Klebsormidium*, depending on which clade is considered to include the genuine *K. flaccidum* the classification of *Klebsormidium* should be rearranged, and one or more new genera should be separated from *Klebsormidium*. More generally, the identity of *K. flaccidum* and *K. nitens* is important in terms of biodiversity and biogeography of the microbial communities of terrestrial habitats. These species are reported among the most common green microalgae in these assemblages (ETTL & GÄRTNER 1995;

LOKHORST 1996; LUKEŠOVÁ 2001; JOHN 2002; RINDI & GUIRY 2004; UHER et al. 2005; ŠKALOUD 2006; MIKHAILYUK 2008) and have been recorded virtually in every region where terrestrial algae have been studied.

For these reasons, the unambiguous placement of *K. flaccidum* and *K. nitens* in molecular phylogenies is a problem of critical importance, which has been remarked in several recent studies (RINDI et al. 2011; ŠKALOUD & RINDI 2013; ŠKALOUD et al. 2014). In his monograph, LOKHORST (1996) designated lectotype specimens for *Ulothrix flaccida* (L 939.67-905) and *U. nitens* (L 939.67-828). Unfortunately both materials consist of small tufts formed by a few filaments, for which detailed morphological observations and DNA extraction are impossible (Willem Prud'homme Van Reine, personal communication). The only possible approach was therefore to select for both species epitype strains, using living material that could be sequenced and linked to molecular phylogenetic clades. The study of MIKHAILYUK et al. (2015) had the great merit of designating such epitypes and fixing the position of these species in the phylogeny of the Klebsormidiales. However, the epitypification of MIKHAILYUK et al. (2015) was based on the characterizations of *Klebsormidium flaccidum* and *K. nitens* given by LOKHORST (1996), and neither of the epitype strains selected by these authors came from the type localities (the strain SAG 2307, epitype of *K. flaccidum*, was isolated from clay soil in a field of beets near Niederkruechten, Germany; the strain SAG 13.91, epitype of *K. nitens*, was isolated from soil at Tekoa, New Zealand). The choice of material obtained from the original type localities and habitats is a procedure strongly recommended for epitypification (HYDE & ZHANG 2008; HUGHEY & GABRIELSON 2012), as it maximizes the chances to obtain authentic material. In the case of the genus *Klebsormidium* we consider this aspect even more important, because several recent studies suggested that ecological preferences play a major role in the distribution and speciation patterns of this genus (ŠKALOUD & RINDI 2013; ŠKALOUD et al. 2014; RYŠÁNEK et al. 2015). So, in this study we carefully looked for material corresponding to the original descriptions of *Ulothrix flaccida* and *U. nitens* in their type localities. We acknowledge that there cannot be the absolute certainty that the strains that we obtained are exactly the same algae used by KÜTZING (1849) for the original descriptions; however, due to the impossibility to sequence the lectotype specimens, this would be inevitably the case with any specimen selected as epitype. We believe that our strains, which were obtained from the original type localities and habitats, have the highest chances to represent the authentic *Ulothrix flaccida* and *U. nitens*.

For *Klebsormidium flaccidum*, three strains (S201, S202, S203) were isolated from a stony street in Strasbourg, the original habitat of the species ("*ad vias lapideas Argentorati*": KÜTZING 1849: 349). The description of *Ulothrix flaccida* is (translated from Latin) "*Ulothrix* green, 6.45-7.52 µm in diameter, with cells as long as wide or twice as long as wide, hyaline; nuclei of cells distant from each other". Though we observed a slight difference in cell dimensions between the three isolated strains (Table 1), their morphology conforms well to Kützing's description (although it is difficult to figure out what Kützing meant for nuclei; we suppose that in this case nuclei might mean the pyrenoids in the chloroplasts) and is also in good agreement with the concept of *Klebsormidium flaccidum* generally established in the literature. This alga is reported as a species with filaments mostly long (>150 cells), thin-walled cylindrical cells, slight constrictions between adjacent cells, chloroplast with smooth margin extending for the whole length of the cell and covering about a half of the lateral wall, and devoid of biseriate parts and false branches. Some disagreement exists in the literature about the range of width of the filaments: 6-9.5 µm in HAZEN (1902); 5-8 µm in MATTOX & BOLD (1962); 5-9 µm in PRINTZ (1964); 5-14 µm in RAMANATHAN (1964) and JOHN (2002); 5.5-6 µm in FAROOQUI (1968); 5.5-7 µm in KOMÁROMY (1976); 5.6-7.4 µm in LOKHORST (1996); 6-9 µm in ŠKALOUD (2006). The dimensions of our strains (having average cell widths 6.3 ± 0.7 µm, 7.0 ± 0.5 µm, and 7.4 ± 0.7 µm, respectively), however, are in the range of width given in Kützing's original description (6.45-7.52 µm). All three strains isolated from the original habitat in Strasbourg were inferred in the superclade C *sensu* RINDI et al. (2011), and are genetically identical to the strain SAG 2307 proposed be represent the epitype of *K. flaccidum* (MIKHAILYUK et al. 2015). Although the strain SAG 2307 was isolated from a sample collected 300 km far from the type locality (Niederkruechten, Germany), our findings corroborate the correct selection of this strain as epitype of *K. flaccidum*.

The unambiguous circumscription of *K. nitens* is more complicated than that of *K. flaccidum*, due to the fact that in the original description the type locality was not explicitly designated. KÜTZING (1849) described *Ulothrix nitens* based on material collected and sent to him by Meneghini, named *Hormidium nitens* in the letter that Meneghini attached to the material (KÜTZING 1849: 349). The original description mentions that the alga was collected from flowerpots, mixed with *Porphyridium purpureum* (BORY) K.M. DREW et R. ROSS ("*in ollis cum Palmella cruenta*"), but does not mention the locality. Giuseppe Meneghini (1811-1889) was an Italian professor of sciences and politician. In the course of his academic career, he was active in the universities of Padua (from 1835 to 1848) and Pisa (from 1849 to 1889); we therefore consider very likely that the collection that he sent to Kützing (and that was used

for the description of *Ulothrix nitens*) was from either of these localities. Considering that *Species algarum* was published by Kützing in 1849 and that the original material of *Ulothrix nitens* must have been received by him sometime earlier, we consider more likely that Meneghini made the original collection in Padua, rather than in Pisa. The botanical garden of Padua is the oldest academic botanical garden in the world (founded in 1545) and still today is located in the original site of foundation. Meneghini must have made large use of it for his academic activity, so we consider it the most likely candidate as collection site of *U. nitens*. This is partly confirmed by Kuzing's remark about the habitat (on flowerpots, mixed with *Porphyridium purpureum*).

Consequently, we isolated in culture numerous strains of *Klebsormidium* from the botanical garden of Padua. Their *rbc*L sequences placed nearly all of them in the clade 12 of ŠKALOUD & RINDI (2013) (=clade E4 of RINDI et al. 2011). The morphology of these algae agrees well with the original description of the species. The protologue of *Ulothrix nitens* (translated from Latin) reports "*Ulothrix* green, with filaments exactly and densely parallel, forming an iridescent membrane when dry, 6.45 µm wide; cells slightly longer than wide or as long as wide; gonidia (nuclei of cells) cylindrical appressed – on flowerpots with *Palmella cruenta*: collected by Meneghini". The strains of *Klebsormidium* that we collected in Padua occurred in small amounts in the field-collected samples and did not form the shiny films mentioned by KÜTZING (1849). However, we collected several specimens from concrete surfaces, including flowerpots (strains F3A, F3B, F3C, F3D, and F3E), for which width of filaments and shape of the cells agree perfectly with the description (concerning gonidia, it is again difficult to understand what Kützing meant; since he mentions the cylindrical shape, we suppose that he might refer to the chloroplasts).

The taxonomic status of *K. nitens* has been questioned by many authors in the past. Indeed, the differences between *K. nitens* and *K. flaccidum* are rather tenuous and in some treatments the two species were not recognized as separate taxa (e.g., CHODAT 1902; FAROOQUI 1968; ETTL & GÄERTNER 1995). Most authors, however, distinguished them based mainly on the diameter of filaments (generally thinner in *K. nitens*) and the form of growth in liquid culture mainly as a superficial film (in *K. nitens*) or as a mass of submerged filaments (in *K. flaccidum*) (KLEBS 1896; PRINTZ 1964; LOKHORST 1996). LOKHORST (1996) reported also that the two species can be distinguished based on some features in culture (easiness of zoosporangial induction, shape of the release aperture of the zoosporangium, pattern of germination of zoospores). ŠKALOUD (2006), however, after an extensive culture investigation performed on 40 strains belonging to the *K. flaccidum*/*nitens* complex, concluded that these

characters in general were not tenable to separate species. Although some characters could be used to define clusters of strains (cell width, aperture type of zoosporangia and microbiotope of habitat), clusters of strains created on the basis of one delimiting character did not correspond with clusters based on other characters (ŠKALOUD 2006). Interestingly, the morphological circumscription of *K. nitens* has apparently changed over time, and significantly deviated from its original description. The taxon was originally described to have filaments 6.45 μm wide (in the protologue, the dimensions were given in historic French length units called lignes, as 1/350‴; KÜTZING 1849). Later on, KLEBS (1896) defined *K. nitens* by having filaments 5.5-7.0 μm wide and his circumscription was then followed by numerous authors (HEERING 1914; PRINTZ 1964; STARMACH 1972). However, in the two most recent taxonomic treatments of the genus *Klebsormidium*, *K. nitens* was defined by having much thinner filaments. FAROOQUI (1968) characterized this alga as having filaments 5.0-5.6 μm wide (although she hypothesized it to represent an ecophysiological morph of *K. flaccidum*). LOKHORST (1996) defined *K. nitens* with cell width 4.7-5.6(-6.5) μm. MIKHAILYUK et al. (2015) followed Lokhorst's circumscription and designated the strain SAG 13.91 as epitype of this taxon. However, this strain has filaments 5.1 ± 0.30 μm wide (ŠKALOUD & RINDI 2013), which clearly does not correspond to the original species description by KÜTZING (1849). In addition, the strain SAG 13.91 was isolated from New Zealand, therefore extremely far from the original sampling site, which (although with the uncertainties mentioned above) almost certainly is in Italy. We therefore conclude that the epitype of *K. nitens* selected by MIKHAILYUK et al. (2015) most probably does not represent the authentic *Ulothrix nitens* described by KÜTZING (1849). For this reason, in our opinion *Klebsormidium nitens* is a suitable candidate taxon for conservation based on a new epitypification. The new epitype would supersede the epitype selected by MIKHAILYUK et al. (2015) and should be a strain isolated from Italy, with morphology corresponding to the original protologue. We suggest our sample F1B (=culture CAUP J 306) would be an appropriate candidate as new epitype for this species.

As general conclusion, we remark the importance of an accurate, well-thought selection of epitype specimens/strains. Although this is true for all algae, it applies in particular to morphologically simple green microalgae, whose huge genetic and ecological diversity is being more and more unravelled. In the future, epitypes will become the essential reference for the definition of many species of morphologically simple algae; their selection should be taken very seriously and handled with great attention, always verifying that the morphology of the material selected agrees with the original protologue. All possible

precautions should be taken to ensure that the epitype really corresponds to the alga of the original description; using material collected in the type locality and isolated from the original habitat will maximize the chance that this is accomplished. An incorrect selection of an epitype has the potential to create major nomenclatural problems and great confusion about the circumscription of a species; such confusion could be spread easily, especially in the case of species of great biotechnological interest, as it the case for some common mirchochlorophytes (HANNON et al. 2010; WU et al. 2014). So far, epitypes have been designated only for a limited number of green microalgae (e.g., KRIENITZ et al. 2010; FUČÍKOVÁ et al. 2013; ALLEWAERT et al. 2015) and many other species that have been described only on morphological basis are still waiting for the fulfilment of this procedure. We hope that epitypes selected by rigorous and accurate procedures will be soon available for many more species.

ACKNOWLEDGEMENTS

We would like to thank Prof. Michael Guiry, National University of Ireland, Galway, for useful and stimulating discussion. The study was supported by a grant from the Charles University Science Foundation (GAUK No. 1544214).

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TABLES

Table 1. Cell dimensions of investigated *Klebsormidium* strains

Strain		cell width (μm)			cell length (μm)		
		average	min	max	average	min	max
	overall dimensions	**7.4 ± 0.7**	**6.4**	**8.8**	**9.6 ± 3.6**	**4.3**	**15.7**
S201	fresh cultures	6.8 ± 0.2	6.4	7.1	11.8 ± 3.5	5.4	15.7
	nutrient-depleted cultures	8.0 ± 0.6	6.6	8.8	7.4 ± 2.0	4.3	10.0
	overall dimensions	**6.3 ± 0.7**	**5.4**	**7.9**	**12.6 ± 3.5**	**5.3**	**20.8**
S202	fresh cultures	5.9 ± 0.3	5.4	6.5	13.7 ± 3.5	7.7	20.8
	nutrient-depleted cultures	6.9 ± 0.6	6.0	7.9	10.9 ± 2.9	5.3	15.7
	overall dimensions	**7.0 ± 0.5**	**5.8**	**8.0**	**12.0 ± 3.3**	**6.1**	**18.1**
S203	fresh cultures	6.7 ± 0.4	5.8	7.4	11.3 ± 3.2	6.1	18.0
	nutrient-depleted cultures	7.4 ± 0.4	6.9	8.0	13.7 ± 2.9	7.5	18.1
	overall dimensions	**6.8 ± 0.4**	**6.1**	**7.7**	**9.4 ± 2.4**	**5.3**	**13.2**
F1B	fresh cultures	7.0 ± 0.3	6.4	7.7	8.7 ± 2.4	5.3	12.6
	nutrient-depleted cultures	6.4 ± 0.2	6.1	6.8	10.7 ± 1.8	6.4	13.2
	overall dimensions	**6.9 ± 0.5**	**6.0**	**7.7**	**10.6 ± 4.1**	**5.4**	**22.7**
F3C	fresh cultures	7.1 ± 0.3	6.6	7.7	9.0 ± 2.5	5.4	14.6
	nutrient-depleted cultures	6.5 ± 0.4	6.0	7.3	13.0 ± 4.8	8.0	22.7
	overall dimensions	**7.4 ± 0.5**	**6.4**	**8.4**	**9.3 ± 2.4**	**5.5**	**14.1**
F8B	fresh cultures	7.7 ± 0.3	7.1	8.4	8.6 ± 2.2	5.7	13.1
	nutrient-depleted cultures	6.8 ± 0.2	6.4	7.2	10.5 ± 2.3	5.5	14.1

FIGURES

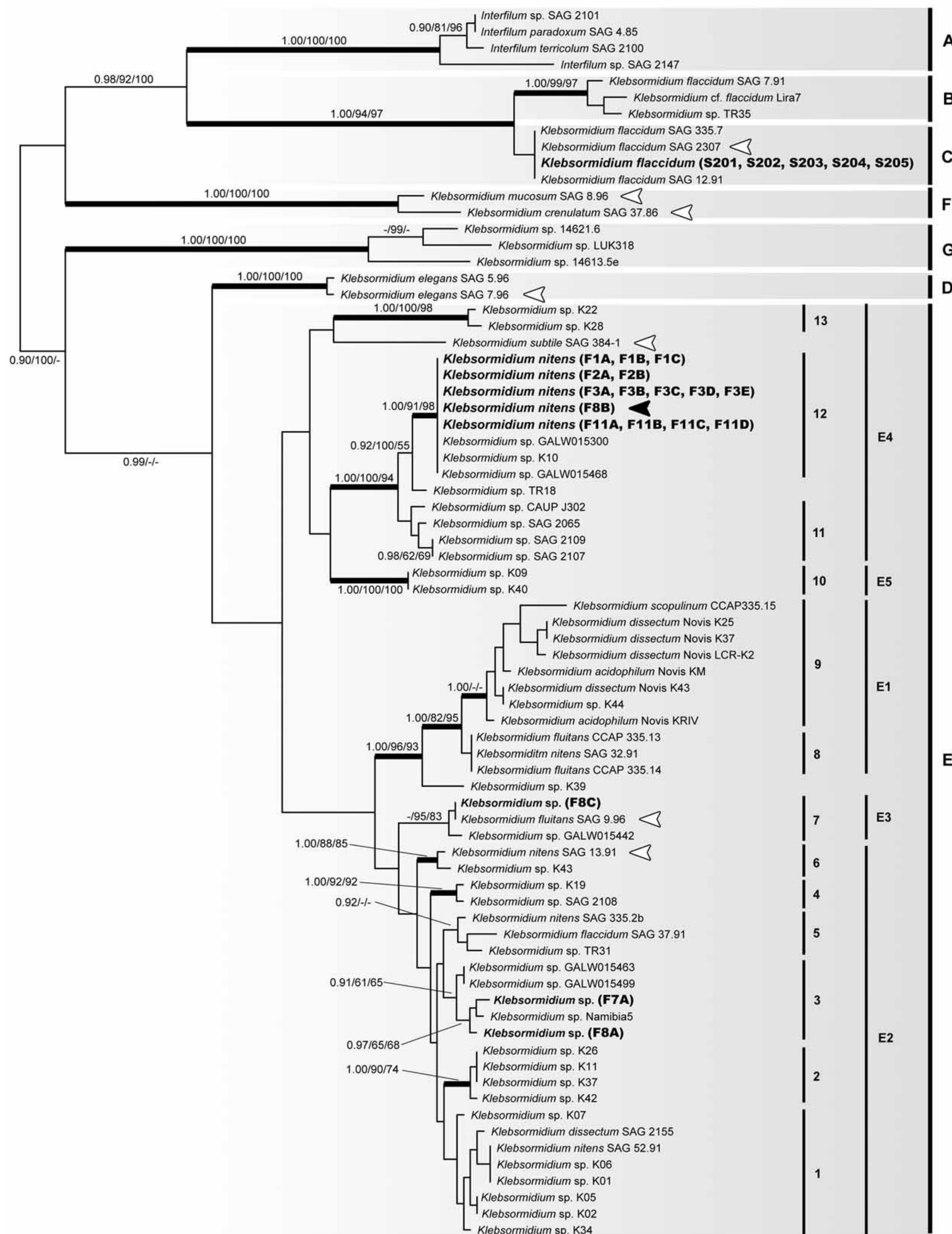

Figure 1. Phylogenetic tree obtained from Bayesian analysis based on *rbc*L dataset, showing the position of investigated *Klebsormidium* strains and their relatives. Values at the nodes indicate statistical support estimated by MrBayes posterior node probability (left), maximum likelihood bootstrap (middle) and maximum parsimony bootstrap (right). The clade numbering (A-G, 1-13) follows Rindi et al. (2011) and Škaloud and Rindi (2013), respectively. The strains selected by previous authors as epitypes of different species are indicated by empty arrowheads. The strain F8B we propose as a new epitype for *K. nitens* is marked by the solid arrowhead.

Figure 2. Morphology of investigated *Klebsormidium flaccidum* and *K. nitens* strain. **A-G,** *K. flaccidum*; **A,** young filament (S202); **B,** mature filament with conspicuous starch grains covering the pyrenoid (S201); **C,** production of a mucilaginous attaching disc (S203); **D,** few-celled filament with a distinct H-shaped wall piece (S202); **E,** empty zoosporangium with a distinct discharge pore (S202); **F,** empty zoosporangium with an indistinct pore (S201); **G,** young filaments grown after a bipolar germination of settled zoospores (S202). **H-J,** *K. nitens*; **H,** young filament with indistinct pyrenoids (F1B); **I,** mature filament with conspicuous starch grains covering the pyrenoid (F3C); **J,** rope-like strands of twisted filaments (F3C). Scale bar: 10 µm.

4. Summary and conclusions

4.1. Microbial biogeography

Although the global biogeographic distributions of several aquatic protists have been documented (Finlay and Clarke 1999, Sabbe et al. 2001, Montresor et al. 2003, Šlapeta et al. 2006, Neustupa and Řezáčová 2007), the biogeography of aeroterrestrial organisms remained largely unclear. In paper 2, we analysed aeroterrestrial algae belonging to the genus *Klebsormidium* to assess their diversity and distribution pattern in mixed forests of the Northern temperate zone. Our extended sampling revealed a cosmopolitan distribution of all of presumably endemic lineages, in a recently published phylogenetic investigation of this genus Rindi et al. (2011). They discussed a limited distribution of several identified clades, based on their investigation strains originating from five continents, for example, the proposed Eastern European superclade B has been identified in North America. Similarly, our sampling revealed the apparent cosmopolitan distribution of superclade C, which was proposed to be restricted to Western Europe. In fact, it is very likely that the presumably limited distribution of some *Klebsormidium* genotypes in our study may be due to their limited sampling. Similar trends were reported by Kristiansen (2008), who stressed that almost all *Mallomonas* taxa originally started as endemics but sooner or later lost this status because of more intensive research. Finally, the results demonstrated that a majority of genotypes represented by several isolates have a cosmopolitan distribution, whereas only a few genotypes were isolated in a single continent. Our study and the work by others (e.g. Bass et al. 2007, Bates et al. 2013) using molecular approaches to examine the distributions of different protistan groups from across the eukaryote tree strongly support what Foissner (1999, 2006) proposed as a moderate endemicity model of microbial biogeography, which puts forward that although some protists may have cosmopolitan distributions, others have rather restricted distributions.

Our investigation in polar regions (paper 3) revealed that the genus *Klebsormidium* exhibits two different distribution patterns common to both macroorganisms and protists. On the one hand, we demonstrated unlimited dispersal and intensive gene flow within one of the inferred lineages (superclade B), corroborating the ubiquitous distribution model of protists (Montresor et al. 2003, Petz et al. 2007). On the other hand, we showed a significant decrease

of species richness towards the poles, i.e. the distribution pattern typical for macroorganisms, such as higher plants and vertebrates (Huston 1994). Therefore, the proposed distinction between the distribution patterns of protists and macroorganisms (Hillebrand and Azovsky 2001, Fenchel and Finlay 2004) cannot be generalized to all organisms. Whereas population genetic investigation shows a clear evidence of a high dispersal capability of superclade B, the absence of several genotypes in the polar regions points to the restricted distribution of the majority of *Klebsormidium* lineages. Such a pattern supports again the moderate endemicity model proposed by the contemporary protistologists (Foissner 1999, 2006, Gast 2015). Consequently, unlimited dispersal of the lineages B should be mediated by wind, because the great majority of *Klebsormidium* species easily disintegrate into fragments containing a few cells (Škaloud 2006). These can then be spread by random events, such as hurricanes or wind currents. Indeed, viable *Klebsormidium* cells have been detected in lower troposphere air samples (Overeem 1937, Sharma et al. 2007). Factors limiting dispersal should be then connected to airborne survival, which is mainly affected by UV radiation and desiccation (Isard and Gage 2001, Figuerola and Green 2002, Sharma et al. 2007).

4.2.Diversity

The genetic diversity within the genus *Klebsormidium* was investigated in detail by Rindi et al. (2011) and Škaloud and Rindi (2013), who identified seven main superclades and 24 well-supported clades. Although we studied *Klebsormidium* in USA, Europe and Japan, we did not discover any novel superclade, but our investigations led to the identification of a large number of new, previously unrecognized genotypes. From a total number of 44 identified genotypes, more than 66% were reported for the first time. Although the majority of published *Klebsormidium* sequences originated from various European isolates, we identified several novel lineages in Wales, including one within the most commonly sampled genotype. This is in accordance with recent research, which use molecular methods that also show hidden diversity (Von der Heyden et al. 2004, Šlapeta et al. 2006, Simon et al. 2008).

Our investigation (paper 3) of newly isolated *Klebsormidium* strains in the polar regions revealed a conspicuously low genetic diversity as compared to the recently published DNA-based diversity assessments. Recently, Mikhailyuk et al. (2015) detected more than 25 ITS rDNA genotypes from 16 different localities in alpine soil crusts. In contrast to the

previously mentioned investigation, we recovered a total of only eight *rbc*L genotypes. Such low genetic diversity could be partly explained by a relatively small number of investigated strains. However, the abundance of *Klebsormidium* in polar regions is obviously very low, which makes very hard to obtain a considerably greater amount of isolated strains. In fact, despite our extensive sampling effort (over 500 samples) in both the Arctic and Antarctica, only 32 strains were successfully isolated. In addition, this low number of found strains could be caused by low abundance, which other studies document as well (Mataloni et al. 2000, Kaštovská et al. 2005, 2007).

4.3.Species concepts

There is (paper 1) the high morphological plasticity related to the environmental factors observed in *Klebsormidium*, an aspect that has complicated many morphological studies. It has been shown that the culture conditions and the age of cultures can significantly affect some morphological characters which were considered useful for species identification for a long time (Škaloud 2006, Rindi et al. 2008). The limited data available indicate that pH conditions may play a major role in this regard: Novis (2006) highlighted a strong effect of pH on the morphologies of *K. acidophilum* and *K. dissectum*, showing that characters such as cell shape, chloroplast shape and amount of granules deposited in the cytoplasm varied considerably in different pH conditions. Variations in pH are also known to affect cell shape in other green microalgae, mainly determining an overall reduction of the cell surface relative to the cell volume (Coesel 1982, Černá and Neustupa 2010).

A connection between the original *Klebsormidium* species descriptions and molecular lineages is necessary for delimitation of new species and for our correct understanding of biogeography, ecology and ecophysiology of particular species. In these days, such information are connected with lineages or just strains (Rindi et al. 2011, Škaloud and Rindi 2013, Karsten and Rindi 2010), but not with the species. Recently, Mikhailyuk et al. (2015) made epytification of 8 species (*K. flaccidum*, *K. crenulatum*, *K. subtile*, *K. nitens*, *K. dissectum*, *K. fluitans*, *K. mucosum*, and *K. elegans*), in which, based on morphologically identified strains from culture collection or natural samples, they connected these strains with specific lineages according to the phylogenetic analyses. However, we prefer collecting samples in type localities and making the taxonomic identification according to the original

descriptions. In paper 5, we focused on *Klebsormidium flaccidum* and *K. nitens*, which we sampled in their original type localities. Based on the original descriptions, we conclude that the designation of the epitype of *K. flaccidum* was correct, whereas the epitype of *K. nitens* (collected on New Zealand, which is tens of thousands of km far from the type locality) was most probably incorrect. Our opinion *Klebsormidium nitens* is a suitable candidate taxon for conservation based on a new epitypification. The new epitype would supersede the epitype selected by Mikhailyuk et al. (2015) and should be a strain isolated from Italy, with morphology corresponding to the original protologue. We suggest our sample F1B (=culture CAUP J 306) would be an appropriate candidate as new epitype for this species.

4.4.Ecology

Our results (paper 1) clearly demonstrate that the capacity to adapt to low pH in *Klebsormidium* is phylogenetically widespread and does not represent a synapomorphy characterizing one or few lineages. In our phylogeny, acid-adapted strains are widely interspersed among congeners living in habitats with neutral conditions. This situation is in agreement with the results of other phylogenetic studies focusing on microalgae from low pH environments, such as unicellular trebouxiophytes (Huss et al. 2002, Juárez et al. 2011), chlamydomonads (Gerloff-Elias et al. 2005, Pollio et al. 2005) and diatoms (Ciniglia et al. 2007). In this genus colonization and adaptation to low pH habitats seem to be frequent events, probably more frequent than in other green algal taxa. Based on the present study and the results of Novis (2006), at least sixteen lineages of *Klebsormidium* have adapted to life in acidic habitats; this is a higher number than for the other genera of green algae that have been investigated to date in this regard. The nature and extent of adaptation to low pH, however, differ in different lineages. Some acid-adapted *Klebsormidium* strains are closely related to strains from non-acidic habitats; some have in fact identical or very similar *rbc*L and ITS sequences to strains isolated from different environments, which is a strong indication of conspecificity. In the cases, we are probably dealing with generalist species with large dispersal and wide pH tolerance, able to survive equally well in acidic and non-acidic environments. Unfortunately, however, physiological data on acid adaptation in *Klebsormidium* are restricted to the experiments of Novis (2006) on *K. acidophilum* and *K. dissectum* from New Zealand.

In the paper 4, we showed that different lineages of *Klebsormidium* are adapted to the substrate on which they originally occur, independently of their evolutionary distance. We found that closely related lineages differed ecophysiologically to the same extent as unrelated clades. Our findings may indicate the widespread existence of sympatric speciation in *Klebsormidium* through ecological divergence, and we hypothesize that this situation is probably common among other taxa of terrestrial algae. The mechanisms of genetic differentiation are not yet fully understood. Specialization to habitats (Gächter and Weisse 2006, Logares et al. 2007), selection pressures (Vanormelingen et al. 2009), and/or persistent founder effects (De Meester et al. 2002) have been hypothesized as important factors contributing to the structure of protist populations. Ecological differentiation facilitates allopatric (De Vargas et al. 1999) and sympatric (e.g. Amato et al. 2007, Weisse 2008, Vanelslander et al. 2009) speciation of protistan cryptic species. Congruent with the recent studies of Fontaneto et al. (2007) and Birky et al. (2010), our data suggest the existence of distinct species units and sympatric speciation in asexual protists.

In summary, in the present study (paper 4) we showed that all strains isolated from sandstone and limestone were able to grow over the range of investigated pH levels but to differing extents. Strains isolated from limestone showed the highest growth rates at pH 7 and pH 8; but these strains grew very slowly at pH 4 and pH 5. Strains isolated from sandstone exhibited two different growth responses. Strains from one of the investigated genotypes showed the highest growth rate at pH 6; whereas strains of the other genotype had almost identical growth rates at all of the investigated pH levels. We conclude that pH is a critical ecological factor that influences the diversity of *Klebsormidium* in terrestrial habitats. Moreover, our data highlighted a distinct ecophysiological differentiation among distantly and closely related lineages, thereby corroborating our hypothesis that the common sympatric speciation of terrestrial algae is driven by ecological divergence.

4.5.Conclusions

In this thesis, we determine that biogeography and ecology have a big influence on the diversity of terrestrial microalgae. We detected the sympatric speciation of terrestrial algae, which is driven by ecological divergence. We clearly showed that pH is a critical ecological factor that influences the diversity of autotrophic protists in terrestrial habitats, and adaptation

to low pH conditions has been developed multiple times independently in the genus *Klebsormidium*. There are many indications that local adaptation to substrate is a very common in this genus (our studies, Škaloud and Rindi 2013), but many studies demonstrated the existence of a strong phenotypic plasticity to temperature (Elster et al. 2008, Karsten and Rindi 2010, Karsten et al. 2015). Some studies focusing on the phenotypic plasticity and local adaptation were performed on aquatic protist (Weisse et al. 2011, Rengefors et al. 2015), however, no such study dealt with terrestrial algae.

Our studies performed in both the temperate zone and polar regions showed the presence of two different distribution patterns supposed to characterize both macroorganisms and microorganisms. We demonstrated an unlimited dispersal and intensive gene flow within one of the inferred lineages (superclade B), which is in agreement with the ubiquity model. However, the majority of *Klebsormidium* clades showed rather a limited distribution. Our research, among the others, led van der Gast (2015) to reject the ubiquity model. Accordingly, we can strictly disagree with the statement introduced by Finlay et al. (1997), who stated that conserving biodiversity at the microbial level has a little meaning. Now, with an increasing evidence for limited distribution of protists, the debates concerning the protection of microorgaisms are more meaningful, in particular if we consider that many new genera and species are still being described (Němcová et al. 2015, Procházková et al. 2015). The importance of protection of protists is also connected with their biotechnological utilization, as many algae are used for production of biofuel (Rosenberg et al. 2008) or for cleaning wastewater (Mallick 2002).

Finally, it is necessary to clarify the taxonomy of *Klebsormidium* species, in particular to link already described species with existing phylogenetic lineages and to introduce new species names to the rest of clades. In these days, information about biogeography, ecophysiology and ecology are usually connected with clades, strains or genotypes, and this led to confusions and inaccuracies in description of those factors in the genus *Klebsormidium*. Our knowledge of the biogeographic distribution and ecological preferences will be very useful for a correct species delimitation. This polyphasic approach (i.e., species delimitation based on a combination of morphology, DNA sequences, physiology and ecology) seems to be promising for identification and circumscription of various species and genera (Coesel and Krienitz 2008, Malavasi et al. 2016).

5. References

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6. Curriculum vitae

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Study

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2010-2012: Master study: Charles University in Prague, Faculty of Science, Dept. Of Botany
Thesis: Biogeografy, diversity and substrate-specificity of aeroterestrial green algal genus *Klebsormidium* (Streptophyta)

2007-2010: Bachelor study: Charles University in Prague, Faculty of science, Dept. Of Botany
Thesis: Biogeography and diversity protist

Stays abroad

2015 October - November: Applied Ecology and Phycology, Institute of Biological Science, University of Rostock, Germany (Ulf Karsten)

2013 March - April: Institute of Botany, University of Innsbruck, Austria (Andreas Holzinger)

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